Testing the Geometric Framework on the Root Foraging Behaviour of *Poa annua*

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Abstract

In the process towards understanding plant root foraging behaviour, much focus has been on root proliferation responses to nutrient availability and on using animal foraging theories such as the marginal value theorem to understand plant root foraging behaviour. A common problem is the lack of generality of these theories and variation is often explained by life history.

The geometric framework was developed as a general animal foraging concept and has been tested on a variety of organisms such as locusts and slime mould. I adapted this framework by replacing the carbohydrate and protein axes with nitrogen and phosphorus. I used *Poa annua* as the model species for the first test on a plant, and I defined the intake target by applying nitrogen and phosphorus at 6 different concentrations and measuring nutrient intake levels. I hypothesised that the plants would defend an optimal intake target and I was able to define the intake target from the data. However, the results showed that there was a phosphorus limitation to reach this target and I found that the plants tend to defend an optimal intake ratio for suboptimal nutrient availability within the boundaries of regulative ability. This provided the optimal nutrient intake ratio which defines the optimal feeding rail. This rail separates the zones in the fitness landscape that determine which nutrient the plant should take up exclusively to reach the optimal feeding rail.

I tested the hypothesis of exclusive feeding and the plant's ability for nutrient self selection with two split root experiments, one to measure root biomass and another to measure carbon allocation using ^{11}C. I found a stronger ability for the plant to regulate phosphorus intake than to regulate nitrogen intake and the labelled carbon experiment showed a direct foraging response of a nitrogen starved plant when provided with a high nitrogen patch. The geometric framework is an invaluable tool for plant research with respect to nutrient cycling and behaviour, and nutrient uptake and regulation mechanisms need to be considered for developing a general plant foraging concept.

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Contents

Part I

Introduction to optimal foraging

1 Foraging theories and models

1.1 Early plant foraging hypotheses

Nutrient limitation to plant growth is a subject that scientists have tried to understand and model for centuries. One of the earliest scientists to present a theory was Ludwich von Liebig. His law of the minimum is explained well by Hooker (1917), the yield of a crop depends on that nutrient that is present in minimum amount or better: “When a quantity is dependent on a number of variable factors and must be a function of one of them, the quantity is that function which gives the minimum value”. In plant nutrition, the elements needed by plants can be substituted for A, B and C, and the maximum amount of possible growth will be the smallest quantity that is obtained by dividing the available amounts by their specific growth values. The law of the minimum simply uses a substance S which is formed from the reaction of A, B and C. The respective reactive values of A, B and C are u, v, and w and p, q, and r reflect the amount of A, B and C that is available. In a given situation that pA, qB and rC are reacting, only an amount of S is formed that equals the smallest of fractions p/u, q/v and r/w (Hooker, 1917). However, Galton’s law of averages involves a process of compensation or integration where the factors with the largest values alleviate the influence of the limiting factors. Individual processes usually obey the Law of the Minimum, whereas on higher organisational levels the principle of integration should be leading. This is easily explained by considering other drivers than nutrients, such as gravitropism or light. When a seed is placed upside down in the soil, these drivers counteract the initial limiting factor which is the reversed position (Hooker, 1917). A third suggestion was made by Liebscher (1895), proposing the law of the optimum which predicts that the growth factor in minimum supply is utilised to a greater extent with increasing supply of the other growth factors (Thomas, 1929). However, according to the results of an experiment on vines from Lagatu and Maume in 1919, a decrease in yield was not due to a limited absorption of the non-limiting elements, but to an increased uptake of these elements therefore causing a nutritional imbalance (Thomas, 1929).

On the assumption that a foraging strategy and morphology is selected for to optimise the investment versus the return of resources, Gleeson and Tilman (1992) built the theory of adaptive plant uptake. So if we can assume that the plant is an optimal forager, the allocation of foraging effort and fitness gain is a consequent of the strategy in which no resource is taken up in excess and therefore all resources limit growth simultaneously. This is valid when there is an interaction in the uptake of two resources. For example, a plant has a carbon cost to take up nitrogen, and there is a nitrogen cost to take up carbon. Also, an excess of nitrogen can be used in the production of phosphatase which is excreted by the plant to mineralise phosphorus in the soil and would thus enhance phosphorus uptake (Olander and Vitousek, 2000). If one resource is low, the other resource will be allocated towards the uptake of that limiting resource, resulting in both resources being equally limiting (Chapin et al., 1987), this leads to what we know as the the multiple limitation hypothesis (Gleeson and Tilman, 1992). The key difference between the law of the minimum and the multiple limitation hypothesis is a matter of the resources or growth factors being taken up independently, or in interaction.

The concept of multiple limitation and optimality was integrated by Bloom et al. (1985) into four theorems. The first theorem predicts that plants take up nutrients at minimal cost and store them until they can be used for maximum return. The second theorem is based on the concept of marginal cost and marginal revenue. A plant should continue investing in growth as long as the carbon, mineral nutrient or water cost per plant mass is less than the increase in plant growth. Additionally theorem 3 predicts that a plant alters its resource allocation such that all resources limit growth equally. Theorem 4 addresses the concept of interaction or substitution, where for example phenolics can be substituted by alkaloids for herbivore defence. Optimal allocation is found when the balance of the resources is equally limiting for every plant process (Bloom et al., 1985). Theorem 3 and 4 differ in the sense that the response is short term and thus plastic (theorem 3) or the response is on the long term, by speciation (theorem 4). Note that neither of the four theorems agree with the law of the minimum.

1.2 Models for animal foraging

Even though Bloom et al. (1985); Gleeson and Tilman (1992); Thomas (1929); Chapin et al. (1987); Hooker (1917) and Olander and Vitousek (2000) discussed their theories on the basis of plant nutrition, much of the model development in nutrient intake and foraging has been based on animal behaviour. Whether we look at animals or plants, both need to invest their resources to meet their growth, reproduction and respiration targets. Animal foraging behaviour has been subject to modelling since the early 60's. Levins [1962, 1963] explored the relationship between the fitness of populations and environmental heterogeneity. MacArthur and Pianka (1966) continued specifying the fitness of populations in terms of optimal foraging behaviour and stated: "determining optimal utilisation of time or energy budgets is very simple: an activity should be enlarged as long as the resulting gain in time spent per unit food exceeds the loss". They subdivide the time per item eaten into a search time and a pursuit and present a graphical method to specify the optimal diet of a predator, in terms of net amount of energy gained from a prey in relation to the energy used in search for that prey (figure 1). The reduction in search time per prey item, Δ S, follows from the assumption that enlarging the diet for N number of prey to $N+1$ equally abundant species reduces the mean search time from $\frac{1}{N}$ to $\frac{1}{N+1}$, hence:

$$\Delta \mathrm{S}=\frac{1}{N}-\frac{1}{N+1} \tag{1}$$

The curve of the pursuit time per prey item, Δ P, is empirically determined and measures the adaptation of the predator species' preference for the prey items. A steeper curve for Δ P would indicate a more specialised predator. This theory led to the compression hypothesis which entails that a decrease in prey abundance should not affect the range of prey items in the diet, but only the range of habitat patch types (Schoener, 1974a).

While MacArthur and Pianka (1966) model the number of prey that should be included in the diet as a function of the resource species' density, Emlen (1966) presents a model for prey species selection under the assumption that the value of the prey species is known. This model too suggests that predator specificity would increase with prey density and vice versa and assumes that natural selection drives the feeding strategy that maximises the net caloric intake per individual per time. Their model incorporates the calories gained as a function of time spent on capture and consumption, calories spent on pursuing prey, the relative prey encounter rate and the likelihood of capturing an encountered prey and the frequency of that prey in the diet. In 1968, Emlen extended this model by integrating distribution curves for prey density.

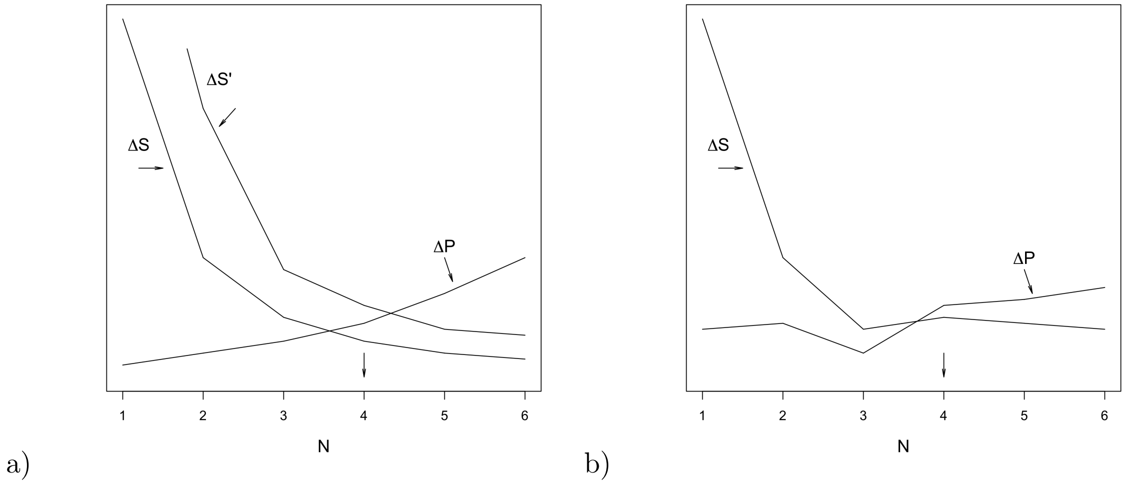


Figure 1: a) Equinumerous resource species. The hypothetical situation is shown, in which the decrease in mean search time (Δ S) and the increase in mean pursuit time (Δ P) accompany the diet increase from N to N+1 prey species. The effect of a doubling in search time is caused by halving the density of each resource species, reflected by doubling the height of Δ S, shown as Δ S'. This leads to a doubling in search time and requires the diet to expand to five prey species. b) Resource species not equally numerous. The curves are no longer monotonic, but the qualitative conclusions still hold. Figures and caption reproduced from MacArthur and Pianka (1966).

A model for food choice and the concept of switching between prey was introduced and tested on snails by Murdoch (1969). Subsequently, Rapport (1971) introduced a geometric model for optimal prey choice depending on the prey availability and the predator's food preference. Again, a change in prey availability may instigate a switch in prey selection. While MacArthur and Pianka (1966) and Rapport (1971) argue that relative abundance of prey determines the optimal diet, Pulliam (1974) proposed a restatement based on the idea that a narrowing of the diet selection would not occur if the competitor were a generalist feeding on many different prey. He suggested that only the absolute abundance of proposed prey is important in determining the optimal diet, because the relative abundance of the alternative prey have no effect. Even though there being various scenarios for optimal diet selection when prey become less abundant, the consensus is that predator specificity increases with food abundance (Pulliam, 1974). However, Evvard (1915) did a study on the dietary self selection of pigs in a free-feeding system, hence a system where food abundance is high. He found that the animals were capable of balancing their diet to match their bodily needs, instead of feeding on a single food type. The same ability to regulate nutrient intake was found in slime-moulds that have no centralised organisational structure to make foraging decisions (Dussutour et al., 2010).

Altogether, two ideas have been addressed at this time: optimality in terms of prey selection, and optimal foraging as time minimisers or energy maximisers. While at this point, energy intake needs to be maximised, the cost currency can be time or energy expenditure. Although usually search time is used, Schoener (1974b) considers two different models, one model that includes only the search time, and another that excludes search time from the total time expense. Where search time is excluded, time only accounts for pursuit, handling and eating prey. The idea is that searching occurs while monitoring mates, other predators and territorial invaders. Because it happens simultaneously, search time itself is not a cost. Either way, the primary task of developing a foraging theory is to choose the currencies to be maximised and minimised, select a cost-benefit function and solve it for the optimum (Schoener, 1971).

1.3 Spatial foraging patterns and decision making

An additional factor to consider is predation risk which may vary between patches and with time. Animals foraging in an environment with the temporal risk of predation need to include decision making on the optimal allocation of anti-predator behaviour for the different risk situations, towards which the risk allocation hypothesis was developed (Lima and Bednekoff, 1999). The theory is that the optimal risk allocation strategy is to reduce vigilance in relatively safe conditions whilst investing in foraging, and increase vigilance and reduce foraging effort in more dangerous situations. Higginson et al. (2012) continued with the development of a model that allows for both variation in food availability, predation risk and environmental conditions. These variables are allowed to autocorrelate in time as to predict future conditions.

A foraging organism has to make decisions on patch choice which is related to patch quality, and while the organism forages on a patch it depletes its resources so the quality decreases. At some point it will need to make the decision whether to stay longer or look for another patch. When it is assumed that patch quality is known, and patch quality and time spent in the patch are independent of the overall food intake and habitat quality, then patch choice can be modelled just like diet choice. When all the patches and their quality are known, remain constant and fitness is a linear function of food intake, it would be optimal to spend all the foraging time in the highest quality patch. When the animal does not know the patch quality of all patches, the optimal foraging strategy depends on the animal's experience and available time (Pyke, 1984). Unknown patch quality before the forager reaches the patch, led to the introduction of the multi armed bandit problem for patch choice (Houston et al., 1988). More practically, different organisms move in a variety of patterns between patches. Describing these movements started with random walk models which were based on Brownian motion (Zumofen et al., 1993). These random walk models are uncorrelated and unbiased (Codling et al., 2008), but when the direction of each step is dependent on the previous step, the random walk is correlated (CRWs) (Patlak, 1953). Other factors increasing the probability of moving in any preferred direction would lead to biased random walk models (BRWs) (Alt, 1980; Codling et al., 2008), while a model used to describe the continuous movement in an unstructured territory would be a mean square displacement model (MSD) (Nouvellet et al., 2009). The direction in biased random walk models for root growth could be driven by gravitropism (Halstead and Dutcher, 1987), galvanotropism (Bayliss, 1907) or hydrotropism (Cassab et al., 2013). Because plant roots tend to grow forward, and are biased in their growth direction by several stimuli, it leads to biased and correlated random walk models (BCRWs). The Levy walk, derived from Levy flight, introduces the time factor and spatiotemporal couplings to the random walk (Zumofen et al., 1993), integrating the correlation aspect of the random walk models. Other examples of stimuli introducing a directional factor to the model, discarding the assumption of unknown patch quality, are prey-taxis (Turchin, 1991; Kareiva and Odell, 1987) or chemotropism (Alt, 1980; Reid et al., 2012). Background nutrient levels in relation to the patch nutrient level (area-restricted search), which is a measure of patch quality (Grunbaum, 1998), complicate the directional drive by chemotropism (Lamb et al., 2004).

Pulliam (1974) introduced movement to the modelling of optimal diets, suggesting that environmental conditions predict predator specificity, which he thought should be the basis of foraging theory. To find the feeding strategy, two assumptions were made: a certain prey abundance and their distribution and a feeding pattern of the predator that defines the encounter rate with each prey type, which is fixed. Additionally, a probability distribution function for search time for every prey type was used. Ritchie (1998) incorporated resource distributions and fractal dimensions in an optimal foraging model, in order to predict the foraging scale that maximises feeding rate. Since optimal feeding is now dependent on prey abundance and distribution, the theory on prey selection is as follows: a prey type x should be included in the diet when the energy gained by pursuing and consuming it per unit time is larger than the energy gained per unit time by searching for and consuming another prey type (Schoener, 1974b). Schoener (1974b) also proposed a model in which there are different types of time expense such as waiting time and foraging time, each with its related energy cost, rather than merely having prey or patch types. This relation should affect the predator's decision making about the length of time to stay in a patch before deciding to travel between patches.

1.4 The marginal value theorem for patch choice

The marginal value theorem was developed by Charnov (1976), as a model for a predator foraging optimally in a patchy habitat. First he conceptualised a typical foraging hierarchy, based on the concepts discussed before. There are different hunting strategies that can be used within the patch, but a predator can always be in only one patch at the time. Figure 2 summarises the hierarchy of the different optimal foraging aspects that have been explored, where most studies have been on prey selection and search mode.

Charnov (1973, 1976) considered the existing models and continued the hierarchy by developing a theory on patch choice, the Marginal Value Theorem. The problem for this theory is that a predator encounters prey in patches and it spends time travelling between patches. The model addresses decision making for patch type and the amount of time spent in the patch, given that the value of the patch decreases with time spent in that patch. Hence the function for food intake per time reaches a maximum (figure 3). Also, the predator is assumed to maximise the net rate of energy intake.

The model:

P_i = the proportion of the visited patches that are of type i (i = 1,2,..., k).

E_T = the energy cost per unit time in travelling between patches.

E_{si} = the energy cost per unit time while searching in a patch of type i.

$h_i(T)$ = the assimilated energy from hunting for T time units in a patch of type i, minus all energy costs except the search cost.

$g_i(T) = h_i(T) - E_{si} \times T$ = the assimilated energy corrected for the cost of searching.

t = the inter-patch travel time.

$T_u = t + \sum P_i \times T_i$ = the average time to use a patch.

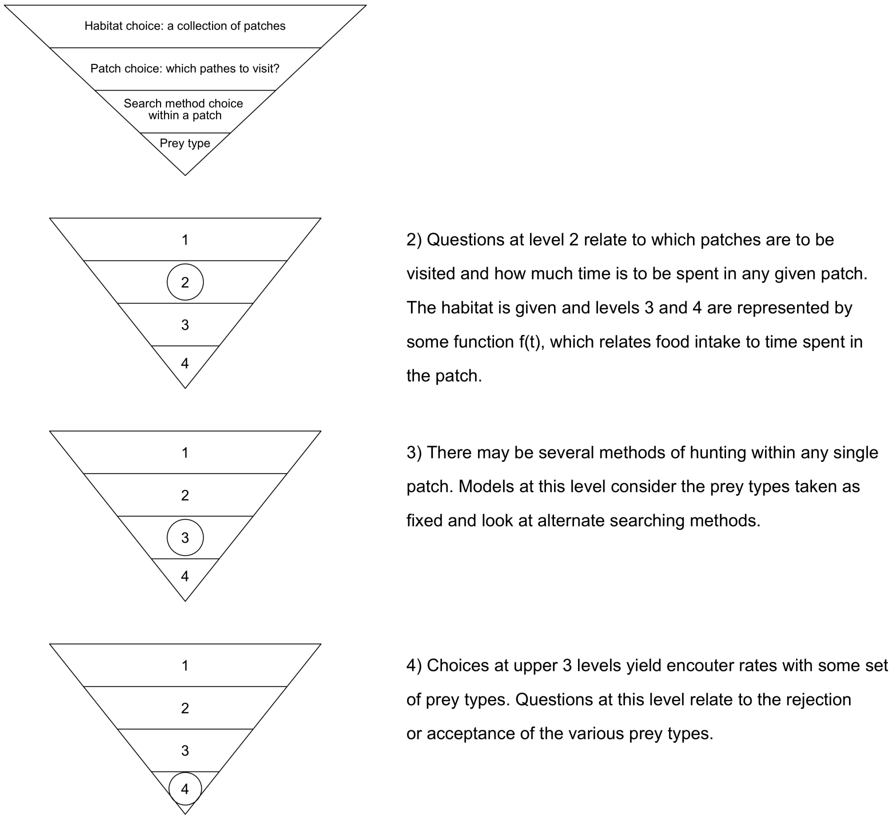
$E_e = \sum P_i \times g_i(T_i)$ = the average energy from a patch.

The net energy intake rate (En) is then given by:

$$En = \frac{E_e - t \times E_T}{T_u} \tag{2}$$

hence:

$$En = \frac{\sum P_i \times g_i(T_i) - t \times E_T}{t + \sum P_i \times T_i} \tag{3}$$



2) Questions at level 2 relate to which patches are to be visited and how much time is to be spent in any given patch. The habitat is given and levels 3 and 4 are represented by some function f(t), which relates food intake to time spent in the patch.

3) There may be several methods of hunting within any single patch. Models at this level consider the prey types taken as fixed and look at alternate searching methods.

4) Choices at upper 3 levels yield encouter rates with some set of prey types. Questions at this level relate to the rejection or acceptance of the various prey types.

Figure 2: The hierarchy of foraging aspects related decision making. The first is habitat choice followed by patch choice within the given habitat. Within the chosen patch the organism can choose between search methods, depending on prey types occurring in the patch. Lowest in the hierarchy comes the rejection or acceptance of prey types. Figure and caption reproduced from Charnov (1973)

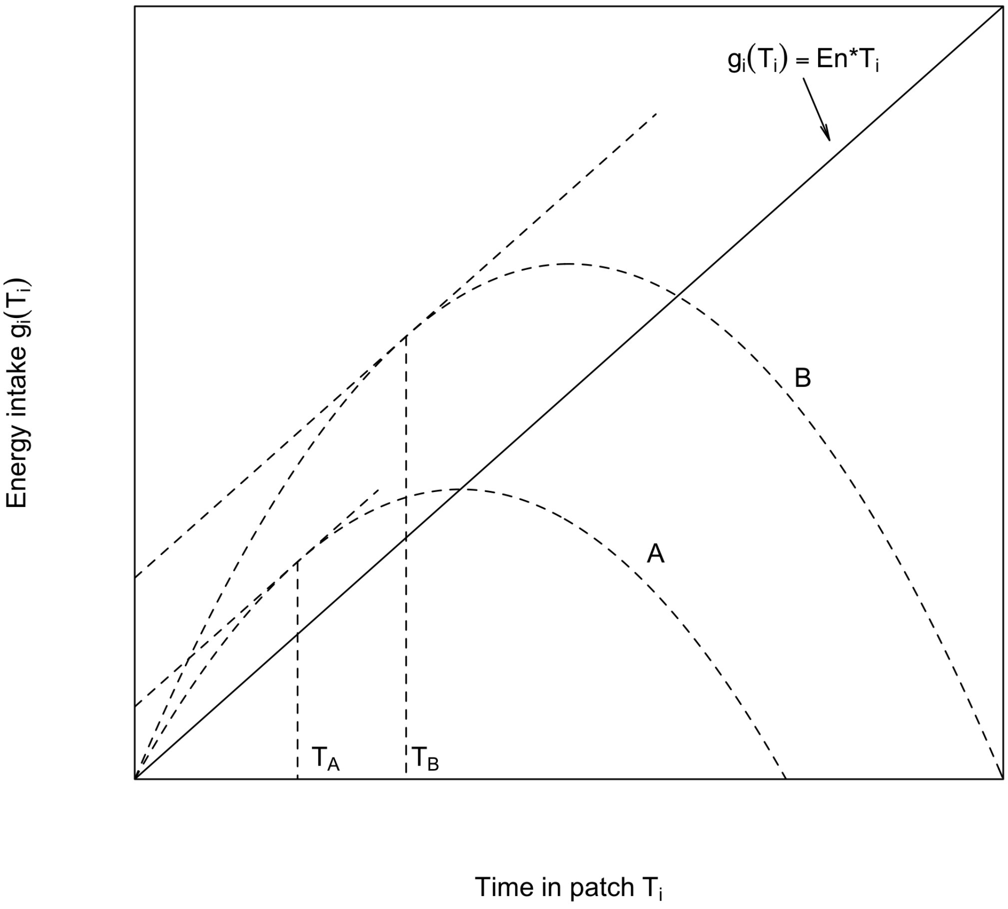


Figure 3: Visualisation of the optimal use of a patchy habitat. For a habitat with two patches the energy intake functions $g_i(T_i)$ are given. The appropriate time to spend in a patch can be found by plotting a line with slope $En*$ and determining the highest line with slope $En*$ tangent to a $g_i(T_i)$ curve. T$_A$ and T$_B$ are the resulting times that should be spent in the patches A and B respectively. Figure and caption reproduced from Charnov (1976).

The predator should control which patches to visit and which not, t is a function of the patches the predator visits and should increase with the number of patches not visited but the time between patches should be independent of the time spent within. So the animal should leave the patch it is foraging in when the the marginal capture rate in the patch ($\partial g/\partial T$) equals the average capture rate for the habitat, irrespective of inter-patch travel time. The average capture rate for the habitat is found when the sum of the proportions of the visited patches ($\sum P_i$) is 1.

Since t does not affect the time spent in a patch, this simplifies equation 3 to:

$$En* = \frac{\partial g_j(T_j)}{\partial T_j} \tag{4}$$

for all i=j

Where $En*$ is the optimal energy intake rate. T_A and T_B can now be found by plotting a line with slope $En*$ and determine where it is tangent to the $g_i(T_i)$ curve, or by solving $En * T_i = g_i(T_i)$ for T_i (Charnov, 1976). Besides depletion rate however, factors like risk allocation and competition should be considered in a model for patch leaving time and patch choice.

A basis for understanding animal distributions as a result of individual decision making has been the ideal free distribution theory (Fretwell and Lucas, 1970). The assumptions are that animals are free to enter each patch and their competitive ability is equal. This was addressed by MacArthur and Pianka (1966) , from their compression hypothesis follows that a predator should only decrease its patch utilisation but not its diet once it is confronted with a competitor. However, this is only valid for a decrease in prey abundance of the preferred prey. If the abundance of the preferred prey is sufficient and the competitor feeds on the lesser preferred prey, there will be no effect. There will only be an effect when the competitor is a generalist and feeding on both prey types, which reduces the abundance of the preferred prey type which causes the initial predator to generalise (Pulliam, 1974).

A restriction to the models described so far is that they are all based on one search mode. Belovsky et al. (1989) developed a blanket model to understand foraging in a wider range of environments. Prey abundance can vary in space and in time, and this model allows for simultaneous search which means that prey are encountered in proportion to their relative abundance in the environment. Contingency models visualise the theory using linear programming where linear segments describe the constraints for multiple food types (figure 4).

The time constraint (T:time) for the amounts (x and y) of two food types (X and Y) are given as:

$$T = (a + b)y + cx \tag{5}$$

if $x \leq (b/d)y$, and

$$T = ay + (c + d)x \tag{6}$$

if $x \geq (b/d)y$

where a is the handling time of the food item Y, b is the search time of the food item Y, c is the handling time of food item X and d is the search time of food item X (Belovsky et al., 1989).

In figure 4a, the segments limit the possible diets that the predator can select, which is the concave surface under the segments. The line segments reflect the the handling time which cause a reduction in available search time. Searching for prey itself does not reduce consumption because the predator can search for both prey simultaneously. Figure 4b reflects the situation where the different prey types are available at different locations or different times of the day. With simultaneous search, the predator encounters prey with a rate related to their relative abundance, but with non-simultaneous search this is not the case. For non-simultaneous search there are two scenarios: the prey are available at the same time but not in the same habitat or the prey are available at different times in the same habitat. Consuming one food type reduces the time for consuming the other, so the time constraint for prey availability in different areas at the same time is given as:

$$T = (a + b)y + (c + d)x \tag{7}$$

which refers to figure 4b.

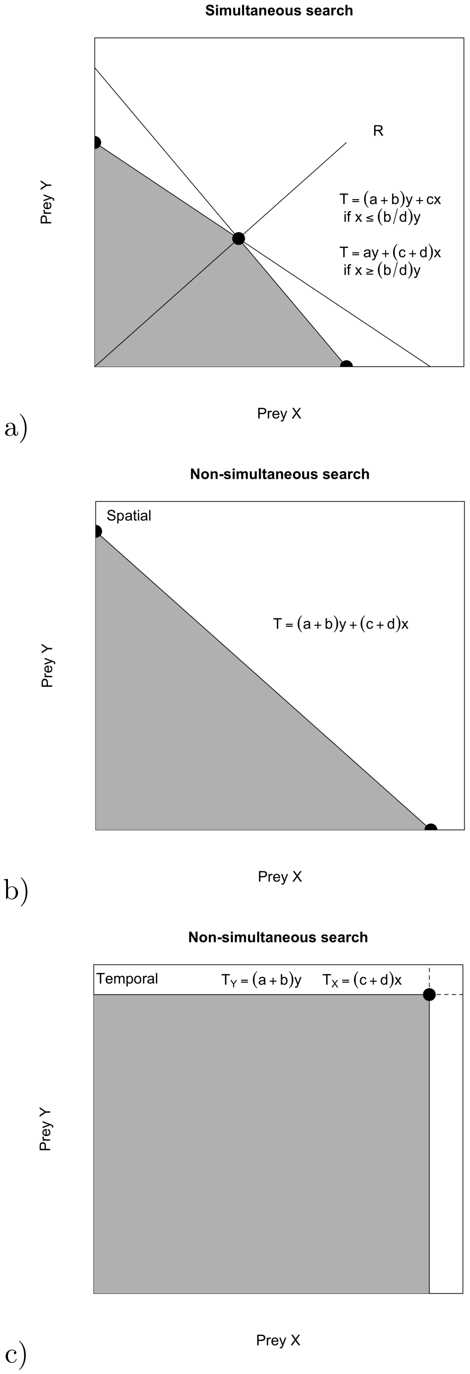


Figure 4: The solid lines are the feeding time constraints (T) for pure prey type distributions in time and space. The constraints are a function of the consumed amount of each food and define the boundary the possible diet combinations given by the shaded regions. Three different models are represented, that is simultaneous search (a), spatial non-simultaneous search (b) and temporal non-simultaneous search (c). The possible optimal diets to optimise energy or nutrient intake are given by the solid dots, they represent the diet that minimises feeding time but do not necessarily maximise the prey consumption. Figures and caption reproduced from Belovsky et al. 1989.

When prey are in the same habitat but only available at different times, the time constraints need to be separated for prey type X and Y , because the animal can only feed exclusively on one or the other prey type. This gives the times constraints as seen in figure 4c:

$$T_Y = (a + b)y \tag{8}$$

and

$$T_X = (c + d)x \tag{9}$$

The consumption of one prey type does not reduce the search and consumption time for the other prey type.

1.5 The geometric framework

One of the factors not considered in previous models like the compression hypothesis or the marginal value theorem, is the current nutritional state of the foraging organism. The requirements should directly affect the directional growth in the random walk models through for example chemotropism. Bertrand's rule states that increased availability of a previously limiting nutrient will first benefit the organism, which will then reach a plateau on which there is no increase in benefit and optimality is maintained through homeostatic regulation, followed by the stage in which an increase in the nutrient concentration will have toxic effects (Mertz, 1981). This is taking the concept of diminishing returns from the marginal value theorem one step further. Bertrand's rule is an important component of the foraging model developed by Simpson and Raubenheimer (1993b, 2012), who present a geometric framework with nutritional targets, dimensions and currencies. Foraging behaviour is an adaptation to minimise the difference between current nutritional state and the intake target, for which the nutrient target is an optimum that is specific for every one of required nutrients. This nutrient target is associated with an intake target, through time, caloric or nutrient cost of processing or assimilating the nutrients that are taken up. There is also a cost of over-ingesting nutrients, if this weren't the case the organism should always maximise rather than regulate intake. In line with this, it was for a long time assumed that Bertrand's rule only applied to micronutrients, and not for macronutrients with caloric value like lipids and carbohydrates (Raubenheimer et al., 2005). However, Raubenheimer et al. (2005) found a fitness compromising ingestion of carbohydrates in *Drosophila melanogaster.* Toxicity of over-ingesting a certain food can for example be caused by secondary compounds in the food (Freeland and Janzen, 1974), but in the case of the fruit fly there seemed to be a direct physiological cost to over ingesting carbohydrates (Raubenheimer et al., 2005). To build the model, the nutrients of interest were placed in a nutritional space which makes up the geometric framework (figure 5). The organism ingests foods that consist of a fixed ratio of the two nutrients. The composition of the available food defines the slope of that food rail, whereas the optimal rail is the line from the origin through the target intake, hence this is also a result of the optimal intake ratio of the two nutrients. With one food available the organism can only reach the target intake when the food rail is on this optimal rail (figure 5a), referred to as line L. When this is not the case (figure 5b), the animal has no option to reach the intake target, and thus has to compromise by either under-ingesting nutrient A (i), over-ingesting nutrient B (iii), or a balance between these two (ii).

When an animal has a choice of two foods consisting of different ratios of these two nutrients, it needs to adopt a strategy of feeding on these to meet the intake target. To find this strategy in an experimental animal, first the intake target has to be established which can be done by providing the animal with various combinations of foods to see which intake levels are preferred.

The intake target can also be derived directly from the organism's physiology. When the nutrient intake is regulated towards the exact target or to the best compromise near the target, instead of it being maximised, the intake target is said to be defended. Hence, when in an experimental setup the intake target is defended by the organism under study, it confirms that there is a regulated and optimal intake for the involved nutrients, rather than intake maximisation. Behaviour however can be adaptive and therefore this intake target can change with life stage or with ecological factors like the seasons or latitude.

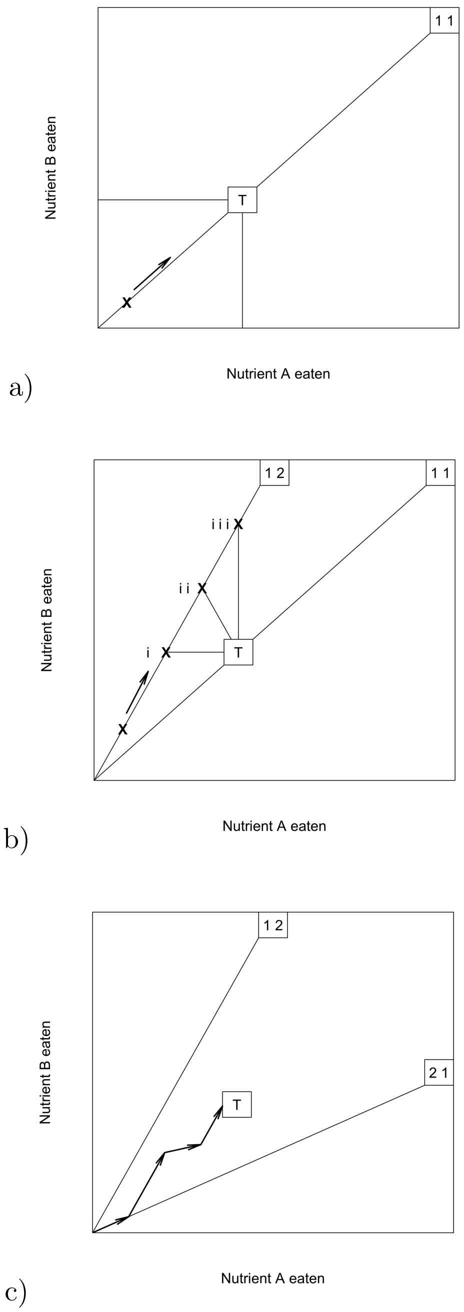


Figure 5: Nutritional planes for intake of two functionally relevant nutrients, A and B. a) The diagonal line is the nutritional rail bearing nutrients A and B in a 1:1 ratio, on this rail lies the intake target (T). The animal's state is shown by X which is in the optimal nutrient ratio on the nutritional rail. It can move towards the intake target following the feeding rail. b) The animal is feeding on a suboptimal feeding rail containing nutrients A and B in a 1:2 ratio. It cannot reach the intake target and has three options of compromise: the animal could chose to reach the intake target of nutrient B which results in a deficiency in A (i), the animal could minimise the distance to the target intake by balancing a deficiency in nutrient A and an excess in nutrient B (ii), or it could chose to reach the intake target of nutrient A by over ingesting nutrient B (iii). c) The animal has two foods available with nutrient A and B in the ratio 1:2 and 2:1. By switching between the two foods it can reach the intake target. Figures and caption reproduced from Simpson and Raubenheimer (1993).

The required protein:carbohydrate ratio may for example decrease when the animal has reached maturity and forages only for energy metabolism. For every situation, the intake target is approached with intake regulation, which may be constrained by food shortage. In this case the animal needs to either spend energy on post-ingestive regulation, or decide to compromise, where the best compromise is driven by natural selection. To find the best compromise for an organism, the same strategy is used as to find the intake target, except that constraints should be added so the target cannot be reached. It is important that the defended intake corresponds with the best performance, would this not be the case then the chosen nutrients may not be the most essential, or the chosen currency for performance does not reflect fitness (Simpson and Raubenheimer, 1993b).

One thing not considered in this geometric framework is the digestive absorption efficiency. This regulates the uptake of nutrients, rather than intake, and therefore the intake strategy is determined by the current nutritional state, the absorption and the intake target.

The intake target defines a location in the nutrient space, around which a linear or quadratic fitness landscape can be shaped to reflect the fitness decline with the deviation from the target. Because the intake mechanisms can differ between the nutrients that make up the nutrient space, the requirement for both nutrients can differ and there may be an interaction in the uptake of the two nutrients, there is a range of different shapes that visualise each situation (figure 6) (Simpson et al., 2004). Figure 6 on the left shows different scenarios for fitness cost landscapes to over and under ingesting protein and carbohydrates. The right figures show the nutritional rails which reflect the composition of each available food. The optimal feeding strategy is derived by connecting the optimal intake points for every nutritional rail. In figure 6a, the cost dependency on the nutrient intake is linear, whereas in figure 6b and 6c the relation is quadratic. In figure 6b however, the cost to over ingestion and under ingestion is equal and thus the fitness cost landscape is symmetrical. However, in figure 6c this shape is not symmetrical, reflecting the situation in which the cost of under ingesting both protein and carbohydrate is larger than the cost to overeating the same amount. Figure 6d represents the situation in which there is an interaction cost additional to the symmetrical quadratic cost relation, which is a result of substitutability of the two nutrients (Simpson et al., 2004).

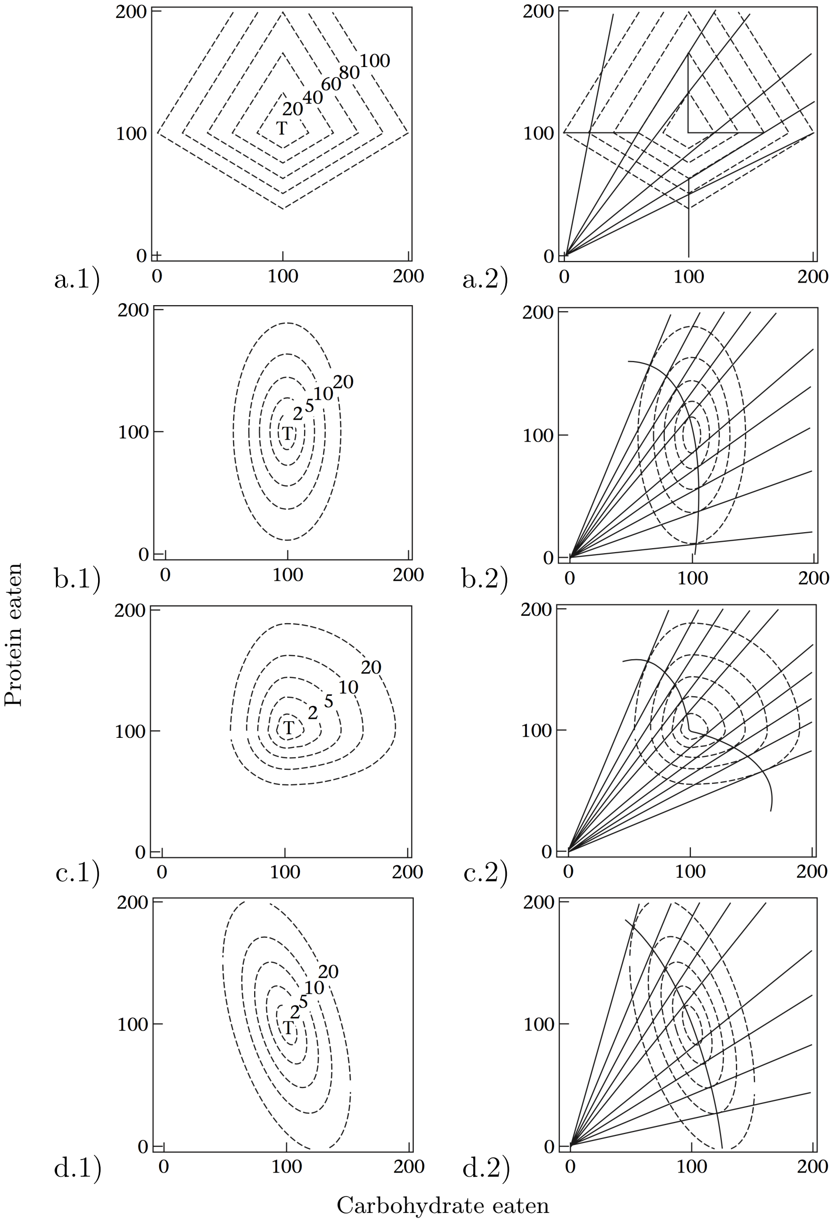


Figure 6: Optimal strategy for the fitness cost. In the plots on the left the fitness contours are shown as the dashed lines and the numbers indicate the fitness cost of deviating from the intake target (T). The feeding rails in the plots in the rigiht row show the protein:carbohydrate ratio of the food. The thicker lines reflect the set optimal strategies in relation to the available food. a) The fitness contours are straight parallel lines around the intake target, which represents a linear cost to deviating from the target in both directions. b) The fitness contours are ellipses and the optimal feeding strategy is also elliptical which represent a symmetrical quadratic cost. c) In every quadrant the fitness contours and the optimal strategy are ellipses representing an asymmetrical quadratic cost. d) These fitness contours are symmetrical quadratic with an interaction cost. The ellipses are tilted which produces a more linear shape for the optimal strategies than in b but the quadratic component of the fitness cost is the same as in b. Figures and caption reproduced from Simpson (2004).

Houston et al. (2011) presented a theory that predicts the sequence of foraging decisions, with the assumptions that there is no cost to switching between foods and there is no relation between state and ecological or metabolic cost. Would there be no interruptions, then there is no preferred strategy to reach the intake target. However, food is not always available and the animal is adapted to interruptions in foraging. This leads to a foraging strategy that is not aimed at moving directly towards the intake target. Equations 10 and 11 describe the state change of nutrient x and y as a function of the uptake of nutrient x and from food type A and the uptake of nutrient x from food type B. $\lambda(x, y)$ is the diet choice strategy of the organism as a function of its state, here the proportion of foraging effort on food type A would be λ, and the foraging effort on food type B is then given by $(1 - \lambda)$.

$$\frac{dx}{dt} = \lambda x_A + (1 - \lambda)x_B \tag{10}$$

and

$$\frac{dy}{dt} = \lambda y_A + (1 - \lambda)y_B \tag{11}$$

$$\frac{d}{dt}R[x(t), y(t)] = \frac{\delta R}{\delta x}x_B + \frac{\delta R}{\delta y}y_B + \lambda\triangle(x, y) \tag{12}$$

where

$$\triangle(x, y) = \frac{\delta R}{\delta x}(x_A - x_B) - \frac{\delta R}{\delta y}(y_B - y_A) \tag{13}$$

R is the reproductive reward, which depends on the organism's state and the diet choice strategy ($\lambda(x, y)$). Hence, the Euclidean distance between the organism's state and the intake target is penalised according to the function in equation 13. To maximise reproductive success instantly, the right hand side of equation 12 must be maximised which is only possible for λ is 0 or 1, unless $\triangle(x, y)$ is 0.

Line L separates the state space into the areas where λ is 0 or 1, so the optimal strategy is to feed on only one food to reach line L and subsequently feed along this switching line to reach the target Houston et al. (2011). Since the slope of line L is $\frac{x_A - x_B}{y_B - y_A}$, the slope increases when the ratio y_B/x_A decreases (figure 7a and b), which causes the forager to feed on food A for a larger proportion of the nutrient space. In figure 7c one food contains both nutrients but has the same nutritional value as if it only contained the one nutrient. Therefore the area for the forager to feed on the food with both nutrients is so large it may not need to feed on the other food. When the food contains both nutrients with equal nutritional value, they do not interact and the slope of the food is on the line L. Figure 7e is the result of adding the same amount of each food to the pure foods of figure 7a. It can happen that two foods are available, but one food contains more of both nutrients which leads the slope of L to be negative and the optimal strategy can not be predicted. Once the forager has reached the switching line, it should feed on the two foods in a ratio between 0 and 1.

When the slope of L is larger than the slopes of both food A and B (figure 7f), the intake target will not be reached when $\lambda = 0$ by following the predicted strategy. For most of the space where $\lambda = 1$, the target can be reached by adopting a different strategy where the least preferred food is consumed before reaching line L Houston et al. (2011).

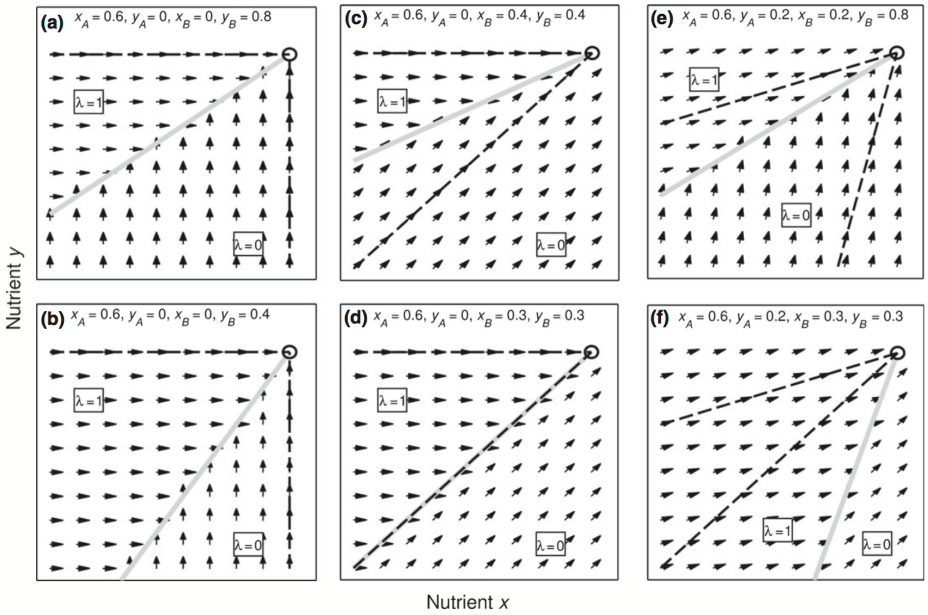


Figure 7: Two dimensional nutrient space with the predicted optimal movements for six different combinations of available food that vary in ratio of nutrient x and y. shown as the black dashed lines. The solid grey line is the optimal feeding rail, L (equation 13), which separates the area where the animal exclusively consumes food A ($\lambda = 1$) and the area it exclusively consumes food B ($\lambda = 0$). The circle in the top right corners is the target intake and the rate the animal changes nutrient state is represented by the length of the arrows. In figure a-d the forager can switch between foods to feed along the optimal feeding rail, but in figure f the forager is not able to stay on line L with the available foods. Figures and caption reproduced from Houston et al. (2011).

However, once the organism has reached line *L,* it is not always possible to consume both foods at the same time. Although figure 5c shows the strategy of switching freely between foods around the optimal feeding rail, the problem of dithering, which would be suboptimal, is not addressed. The reason dithering is suboptimal, is that the time and energy spent between two foods provide a cost. Houston and Sumida (1985) presented a positive feedback model for which the idea is that feeding on one food or the other is associated with both the increase and decrease of the related tendency. A solution would be to have the positive effect rising rapidly up to the maximum, which causes the tendency for this activity to be higher than the tendency for the previous activity. Only when the activity stops it ends the positive feedback (Houston and Sumida, 1985). However, the drivers that define the tendency to feed on one food or the other may interact. Therefore the positive feedback model for switching between activities was extended by Marshall et al. (2015), where they introduced cross inhibition between tendencies, and the strength of the cross inhibition varies with the strength of the tendencies. Although the positive feedback model extended with cross inhibition was found to be better than without the cross inhibition, they assumed that each food being foraged for only consists of one nutrient. Relaxing the assumption of a single nutrient and introducing a second nutrient provides a parallel with the prediction of activity sequence by Houston et al. (2011). Going back to the assumption that there is no cost related to switching between activities, and if the assumption that food sources are not available indefinitely is reasonable, there is a clear optimal strategy that can be tested. Raubenheimer et al. (2005) provided some users guidelines to build the geometric framework for any organism of interest, and with this the model from Houston et al. (2011) for the optimal the foraging strategy can be tested, provided that the functional behaviour meets the assumptions.

2 Plant nutrient uptake mechanisms

2.1 Basic plant physiology

Plant organs Flowering plants are generally built up of a root, stem and leaves, with each having a particular cell structure and internal organisation, specified to its function. The main function of roots is to provide anchorage, store nutrients or starch, and absorb and transport water and nutrients to the other plant parts while transporting photosynthates down the root system. The stem also needs to transport water and nutrients from the roots to the leaves and translocate photosynthates, nutrients and signalling hormones in various directions. The leaves collect light and use this energy to produce sugars. Evapotranspiration also occurs in the leaves, which cools the plant and drives a water flow with solutes upwards through the plant, aided by capillary pressure.

Organ cell structure The root system has a structure of xylem in the centre for bulk flow, and the phloem structure for photosynthate transport. The xylem and phloem are surrounded by a pericycle, endodermis, the cortex and the epidermis, through which water and nutrients from outside the root need to travel to reach the xylem. Root hairs are extensions of root epidermal cells and increase the root surface area to make contact with the soil solution. In the stem, the xylem and phloem are arranged around the vascular cambium from which they develop around the pith in the middle. The outer ring consists of the cortex and the epidermis. The xylem and phloem continue through the leaves, enclosed by parenchyma cells that make up the mesophyll. The upper layer of the mesophyll is the upper palisade layer where most of the chloroplasts are located. The mesophyll is enclosed by the upper and lower epidermis in which the stomata are located. Through the stomata water evaporates and carbon dioxide is taken up.

The plant cell Plant cells have specialised functions, which is reflected in their composition. Generally, a cell contains a nucleus and cytoplasm which contains the various organelles. The cytoplasm is surrounded by a plasma membrane and a cell wall. The cellulose cell wall and the plasma membrane are both semipermeable, allowing water and small molecules to pass freely. Only a secondary cell wall lined with lignin is not permeable to water, to prevent leeching during transport through for example the xylem. The plasma membrane also contains aquaporines, water selective channels formed of integral membrane proteins, which allow water to move faster in and out of the cell than through the lipid bilayer. Adjacent cells are connected by plasmodesmata, tubular extensions of the plasma membrane, this allows a continuous connection of cytoplasms which forms the symplast. There is also a continuous connection of cell walls which lines the intercellular space, the apoplast. From the root tip or root hairs, water can travel to the xylem through the symplast system, the apoplast system and by passing straight through every cell membrane. Hence these cell walls are semi-permeable, while the cells responsible for bulk flow have a waterproof lining.

2.2 Nutrient uptake kinetics

General uptake and transport Most soil particles have a negative charge on their surface, binding mineral cations like K^+, Ca^{2+} or $NH_4^+$to form a cation exchange complex. The pH of the soil solution is important for nutrient uptake, because H^+ions can displace the bound cations in the cation exchange complex which makes them available for uptake. Since the cell membrane is semipermeable, small molecules can be taken up passively with the movement of water from the rhizosphere into the roots, or down a chemical potential gradient. Larger molecules can travel into the plant when the osmotic potential outside the root system is lower than inside, active transport is required which is provided by the hydrolysis of ATP. A plant needs essential elements and energy from light to grow and maintain metabolism. Some elements are directly assimilated once they enter the roots, and transported as an organic molecule, others stay in the soil solution and move with the water transport through the xylem. Essential elements for plants are mineral nutrients, hydrogen, oxygen and carbon. Nitrogen and sulphur are assimilated by reduction and oxidation, forming covalent bonds with carbon. Phosphorus, silicate and boron form phosphate, borate and silicate esters by covalently binding to the hydroxyl group of an organic molecule. These elements are used for storing energy as ATP (adenine tri-phosphate) or NADP(H) (nicotinamide adenine dinucleotide phosphate), or for supporting cell wall structure. A small group of elements occur in the plant in ionic form, often to maintain osmotic potential and as electrostatically bound cofactors to enzymes. The metals, which are important for electron transfer in redox reactions.

Transport also occurs passively down a chemical potential gradient, or actively when compounds need to go up a potential gradient or when they cannot cross the membrane. Transport proteins are embedded in the plasma membrane, providing the function of channel, carrier or pump. Channels are selective pores through which transport is passive. The size and electric charge of the pore determine the specificity and mainly transports water and ions. The pores can open and close in response to for example a voltage signal, ion concentrations or pH. Some channels only allow anions to travel one way, requiring a different mechanism to go back. Only certain compounds can bind to the specific site of the carrier protein, which can therefore be highly selective. When the substance binds to the protein the protein changes shape, together they move into the cell where the substance is released. Transport with the aid of a carrier protein can be either passive or active and is relatively slow compared to transport through a channel because substances are translocated one by one. There is a distinction between primary and secondary active transport. Primary active transport is directly associated with the energy source like ATP-hydrolysis, the absorption of light by a carrier protein or an oxidation-reduction reaction. The primary active transport through a carrier protein (pump) requires an energy releasing event to facilitate the energetically uphill transport of the substance. Pumps are in the family of transporters known as the ATP-binding cassette. They are usually an H^+- ATP-ase generating an H^+gradient across the plasma membrane. Secondary active transport is the coupling of the uphill transport of a solute to the downhill transport of another (anti-port) or the simultaneous transport of two solutes in the same direction (symport).

Nitrogen assimilation There is a specific set of essential macronutrients that plants need and to understand plant root foraging behaviour it is straightforward to focus on the two nutrients in highest demand, which are nitrogen and phosphorus. Plants need nitrogen primarily for proteins and nucleotides and is taken up as nitrate or ammonium. Oxygen availability in the soil determines the level of nitrification on the root surface. Hence, in aerated soils, and when the roots release oxygen via their aerenchyma, most available ammonium is mainly taken up as nitrate. This nitrification of the root surface causes the soil to acidify (equation 14), which in turn affects the availability of nitrogen and other nutrients.

$$2NH_4^+ + 3O_2 = 2NO_2^- + 2H_2O + 4H^+ \tag{14}$$

Nitrate that enters the root can be assimilated immediately, or be transported to the leaves where it is reduced to nitrite and subsequently to ammonium. Ammonium that is taken up directly, is transported to the leaves with ammonium transporter proteins (AMTs). Subsequently ammonium is assimilated into amino acids, this requires carbon skeletons which are produced by photosynthesis. During the vegetative state of the plant, amino acids are transported from the roots to the leaves, whereas the proteins in the seeds are for the largest part derived through the degradation of existing leaves during senescence (figure 8) (Xu et al., 2012). Nitrogen uptake efficiency depends both on root architecture and carbon availability (Xu et al., 2012). Lateral root initiation is stimulated by localised ammonium supply, and elongation of these lateral roots is stimulated by nitrate availability. Low sucrose levels in the soil stimulate the activity of high affinity nitrate transporter (NRT2.1), whereas its activity is reduced under high sucrose availability.

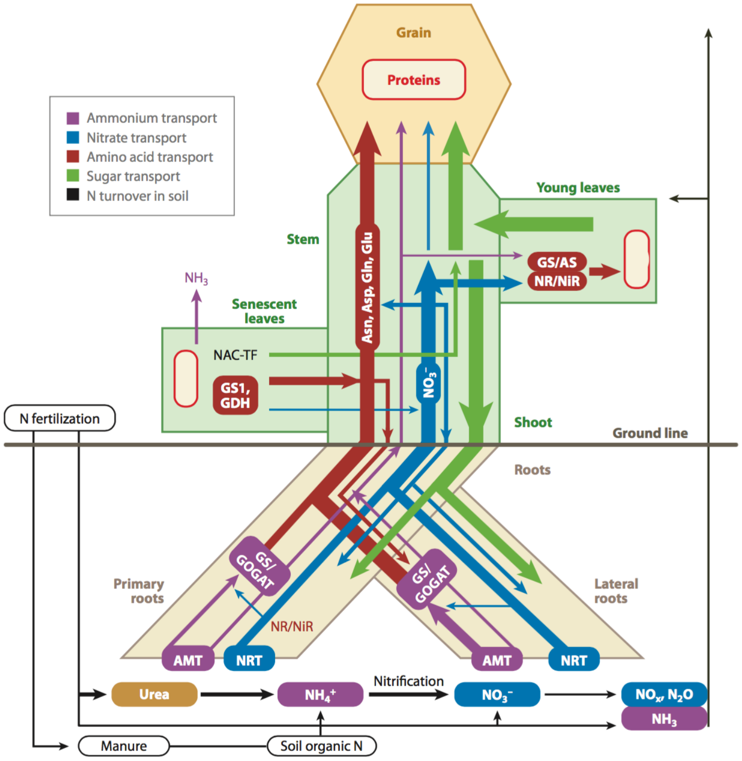


Figure 8: The routes of N uptake from the soil, mainly as ammonium or nitrate. The schematic shows the uptake, transport, assimilation and remobilisation. The relative amounts of nitrogen and carbohydrates are reflected by the thickness of the arrows. Terms: AMT = ammonium transporter; AS = asparagine synthetase = Asn = asparagine; Asp = aspartate; GDH = glutamate dehydrogenase; Gln = glutamine; Glu = glutamate; GOGAT = glutamine-2-oxoglutarate aminotransferase; GS = glutamine synthetase; NAC-TF = NAC family transcription factors; NiR = nitrite reductase; NR = nitrate reductase; NRT = nitrate transporter. Figure and caption reproduced from Xu et al. (2012).

The exact nitrogen use efficiency, remobilisation ability, the number of high affinity nitrate transporters and other morphophysiological traits, is dependent on the plants life history (Xu et al., 2012). There are for example profound differences between the number of genes and the family structure of nitrate transporters (NRTs) between monocots and dicots. The variation in transporter protein number and family results in different preferences for nitrate or ammonium uptake between plant species.

Figure 9 shows the relation between nitrate uptake by NRTs, and ammonium uptake by AMTs in the plasma membrane (Xu et al., 2012). Ammonia is taken up as either NH_3 or NH_4^+ by a uniporter, or as NH_4^+ together with H^+ through a symporter. Nitrate is transported together with $2H^+$, after which it is reduced to ammonia in a plastid and subsequently assimilated into amino acids through the GOGAT cycle (figure 8).

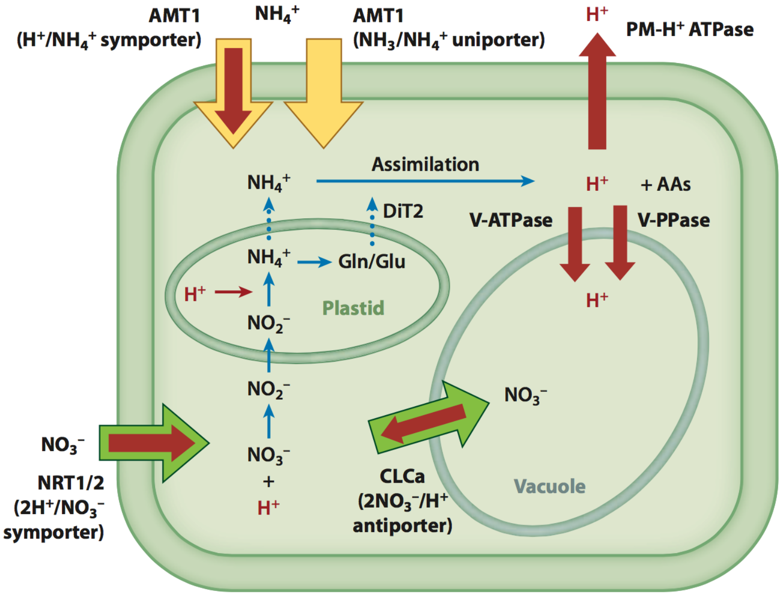


Figure 9: Ammonium and nitrate uptake, cytosolic pH and their interaction. The plasma membrane (PM) ammonium transporter, AMT1, functions as the ammonium uniporter or symporter with H$^+$. The nitrate plus H$^+$ symporters of the NRT1 and NRT2 family also located on the plasma membrane. The tonoplast separates the vacuole from the cytosol, on this tonoplast the CLCa nitrate proton antiporter is located to transport nitrate from the cytosol to the vacuole. This ammonium and nitrate influx into the cytosol and the nitrate influx into the vacuole causes a decrease in pH of the cytosol, subsequently these protons are pumped out of the cytosol or into the vacuole through the H$^+$-ATPase on the vacuole or plasma membrane (AAs = Amino acids). The green arrows represent the nitrate flux, yellow arrows represent the ammonium flux and red arrows represent proton fluxes. The small blue arrows show the ammonium assimilation and nitrate reduction pathway. The dashed blue arrow show the ammonium ion and glutamine (Gln)/glutamate (Glu) efflux from the plastid, the small red arrows show the H$^+$ that is needed for the nitrite reduction. Figure and caption reproduced from Xu et al. (2012).

Phosphorus assimilation Phosphorus is a critical macronutrient needed for energy generation, nucleic acid synthesis, glycolysis, membrane synthesis, photosynthesis, respiration, redox reactions, signalling, carbohydrate metabolism, enzyme activation and nitrogen fixation (Taiz and Zeiger, 2006). Root architecture is important for phosphorus acquisition, and is highly responsive to phosphorus availability which is generally attributed to the fact that phosphorus is relatively immobile in the soil. Williamson et al. (2001) found in *A. thaliana* that low phosphorus availability caused branching through the development of lateral roots and that the development of root hairs increased the uptake surface. Also primary root length can be influenced and cluster formation can occur. The exact level and manner of plasticity is species dependent, therefore it is more important is to understand how it functions, and not to which extent, so that general concepts can be analysed on various species. A series of genes and a class of transcriptional regulators have been identified that affect root architecture in response to nitrogen and phosphorus availability, extensively reviewed by Briat et al. (2015).

The total amount of phosphorus in soil is not fully available to the plant. It occurs for 80% in organic form and the remaining 20% inorganic phosphorus (Pi) precipitates easily or binds to soil particles (Schachtman et al., 1998). Since phosphorus has low solubility, it mainly moves through the soil through diffusion, rather than bulk flow. The availability of Pi is usually only up to 10 µM in the soil solution and because the demand is much higher, Pi needs to be transported up a concentration gradient. Inorganic phosphorus is taken up as as orthophosphates, $H_2PO_4^-$ or $HPO_4{}^{2-}$, with a preference for $H_2PO_4^-$ when soil acidity has pH 4.5-5 (Taiz and Zeiger, 2006). The rhizodermal cells take up the Pi into the symplasm, after which some moves into the cell cytosol where it is bound to form ATP (adenine triphosphate). Some of the phosphorus is used in the biosynthetic pathways of P-lipids, DNA and RNA. Cell homeostasis is regulated by Pi storage in the vacuole, and when internal phosphorus concentration are high, some can be excreted back into the soil solution. Much of the Pi is transported through the symplasm directly to the xylem, through where it is transported to the shoots and leaves. Because of the higher concentration in the cell and the negative membrane potential, active uptake occurs, usually in co-transport with a cation. Inside the cell, there is generally a positive electron potential between the cytoplasm and the vacuole, allowing passive transport into this organelle, although active transport is possible (Taiz and Zeiger, 2006). Membrane transporters can be classed in two categories, low affinity and high affinity transporters. The high affinity system is either increased or de-repressed when phosphorus availability is low, while the low affinity system has low activity and is constitutive (Schachtman et al., 1998). The genes of the phosphate transporter gene family can be divided into two groups, one group for the expression low affinity transporters, and another group for the expression of high affinity transporter proteins. The expression of high affinity transporter proteins tends to be up and down regulated according to phosphorus availability, while the expression of low affinity transporters remains constant.

The Pi concentration of the cytoplasm is kept between 5-10 µM if possible, but the concentrations in the vacuole are highly variable. If the soil concentrations are low, a plant could grow more roots to explore the soil space and depletes any Pi stores of the vacuole. To prevent intake up to toxic levels at high availability, uptake through the transporter proteins is reduced, phosphorus can be stored in organic compounds and up to 70% of intake can be excreted (Bieleski, 1973). Retranslocation from older leaves to the roots is through the phloem and can also occur through organic compounds. Curiously, 50% of this down transported Pi may directly be transported up through the xylem again (Schachtman et al., 1998).

Plants have also developed a mechanisms to conserve the use of P to ensure a high phosphorus use efficiency (PUE). It consists of a carbon metabolism mechanism that reduces P requirements or remobilisation of internal phosphorus. Phosphorus uptake can be increased by stimulating lateral root growth and surface area while decreasing primary root growth, by the activation of Pi transporters and phosphatase, and by organic acid secretion. Therefore the root architecture is important for phosphorus uptake, and a high relative root surface area is related to higher phosphorus uptake. Mycorrhiza increase the root surface area and mobilise phosphorus, while the roots can excrete large amounts of organic acids which acidify the soil and can aid mobilising P.

The transport proteins for phosphorus uptake belong to the PHT and PHO1 families 10. At the root-soil interface they are specialised to extracting µM amounts of inorganic P from the soil solution, which is necessary for transporting the negatively charged molecules against a concentration and electrochemical gradient. $H_2PO_4^-$is transported through the membrane with a symporter together with a cation to prevent hyper polarization of the plasma membrane. The cation that aids the transport through the symporter is usually an H^+ atom, which causes acidification of the cytoplasm . This symporter transport can probably also occur with an Na^+, which is shown in green algae and fungi studies (Briat et al., 2015). Low affinity phosphate/H^+symporters are found in the intercellular organelles where phosphorus concentrations are higher (Briat et al., 2015). The gene expression responses to Pi deficiency are mainly related to PHR1, via the pathway described in figure 10.

Besides regulating the expression of Pi transporter protein transcription factors, PHR1 is also linked to the gene expression of transcriptional regulators of sulphur, ferrum and zinc homeostasis and transport (Briat et al., 2015). This leads to an interaction in the uptake of the various nutrients and is an indication of the plants ability to maintain stoichiometry of certain elements.

Interaction Enzymes produced by plants and soil organisms can mineralise organically bound nutrients that are otherwise unavailable to the plant. To increase nutrient uptake under low nutrient availability, the plant can increase the production and excretion of these enzymes(Gusewell, 2004). Regulation of enzyme production becomes complex where the N and P cycles interact. Olander and Vitousek (2000) measured acid phosphatase and chitinase (N-acetyl ß-D-glucosaminide) activity in soil across a chronosequence in Hawaii where N and P availability varies substantially among sites and long term fertiliser plots had been maintained for over 4 years. They found a high phosphatase activity at all sites and chitinase activity decreased significantly as age and N availability increased across the chronosequence which indicates chitinase is an important enzyme for nitrogen uptake. Phosphorus addition suppressed phosphatase activity, while N addition increased phosphatase activity at the young, N-limited site which indicates an increased demand for phosphorus at increase nitrogen availability. In contrast, N addition repressed chitinase activity only at the N limited young site, and P additions had no effect on chitinase activity. These results suggest that the regulatory relationship between nutrient supply and nutrient mineralization are asymmetric for N and P, and that the differences could help to explain differences observed in patterns of N and P availability.

The relative availability of nitrogen and phosphorus in the soil is reflected in the N-P ratio of the plant biomass and influences vegetation structure (Gusewell, 2004). When a plant is equally limited by N and P it is said to have an optimal N-P ratio and this ratio depends on the species, life stage, growth rate and varies between plant parts.

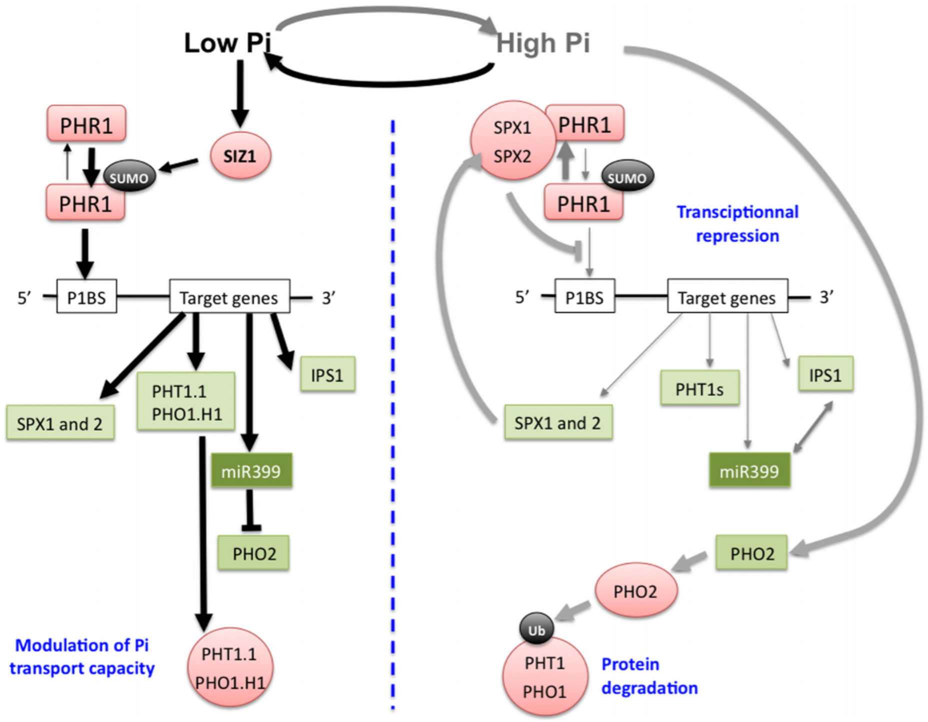


Figure 10: The regulatory pathways for the adaptation of a plant to Pi deficiency. The left panel shows the low Pi nutrition condition, the transcriptional activation of a set of genes needed for Pi uptake by the roots (PHT1, PHO1), occurs through binding of the transcription factor (TF) PHOSPHATE STARVATION RESPONSE 1(PHR1) to its cis-target present in the promoter region of these genes. SIZI stimulates PHR1, which seems to be important for PHR1 activity because under low Pi conditions the Pi-deficient regulated genes stop being induced in siz1 mutants (Miura et al., 2005), however, the mechanism of this regulation is unknown. Post-transcriptional regulators of Pi transporter proteins (PHT1.1, PHO1.H1) are also transcriptionally up-regulated through PHR1 activity under Pi-deficiency. The miRNA miR399 down regulates the ubiquitin E2 conjugase PHO2 responsible of the ubiquitination of PHT1 and PHO1 proteins to target them for proteasome degradation. Under high Pi miR399-dependent inhibition of PHO2 can be titrated through RNA mimicry via its appariement to IPS1, a non-coding RNA up-regulated by PHR1 under Pi deficiency. Under high Pi nutrition conditions (right panel) PHR1 target genes are transcriptionally repressed and PHO2 expression is activated promoting Pi transporters degradation. This transcriptional repression under these conditions is mediated through Pi sensing of nuclear SPX proteins which interact with PHR1 via their SPX domain in Pi-dependent manner in order to inhibit PHR1 binding to its P1BS cis-acting sequence found in the promoter region of Pi responsive genes. The green boxes show the transcripts, red shapes represent proteins, the black shapes are post-translational modifications and arrow thickness is proportional to the strength of the considered flux. Figure and caption reproduced from Briat et al. (2015)

The general ratio range is between 10 and 20, outside this range one or the other nutrient is considered limiting. Biomass N-P ratio's are a result of the uptake and loss of nitrogen and phosphorous (Gusewell, 2004). Intraspecific variation in the ratio is large and the N-P ratio varies by 25% within species growing on the same site. Variation in N-P ratio in grasses is mainly determined by phosphorus intake because nitrogen concentration tends to be relatively stable. Because of the ability of the plant to regulate intake, a 10-fold variation in the ratio of N-P supply only results in a 2 to 4-fold variation in intake ratio (Gusewell, 2004). The relationship between supply ratio and intake ratio can be defined by the regulatory coefficient H which is the inverse slope of the regression line fitted to log-transformed variable and the degree of regulation depends on light intensity and on the overall nutrient supply. Toxic effects are more likely to occur in plants adapted to nutrient poor soils that have a lower ability to downregulate intake. The signalling for uptake regulation is primarily sensitive to the composition of the phloem (Zhang, 2000; Gusewell, 2004). The regulation is two-way, with a down regulated uptake of phosphorus in nitrogen deficient plants while up regulating nitrogen uptake and vice versa which is in contrast to the findings of Olander and Vitousek (2000). Ammonium uptake can be down-regulated less effectively than nitrate uptake so that the relative amount of nitrogen uptake in phosphorus deficient plants is high (Schjorring, 1986). High phosphorus concentration in the phloem causes either a down regulated uptake or it can be stored as polyphosphate in the vacuole of stems, roots and seeds.

Uptake regulation is limited to nutrient availability, and there are also limitations to down-regulating nutrient intake. When downregulation is insufficient toxic levels in the plant tissue can occur (Howitt and Udvardi, 2000). Each root tip has a range of sensory mechanisms that allow it to respond to environmental signals (Filleur et al., 2005). The MADS (MCM1, agamous, deficient and SRF) box gene (ANR1) is involved in modulating the rate of lateral root growth affected by nitrate concentration of the soil solution (Zhang and Forde, 1998; Walch-Liu et al., 2006). When the supplied ammonium is oxidised to nitrate by aerobic bacteria, post transcriptional regulation of uptake can occur through the activation (or lack thereof) of the ANR1 protein (Filleur et al., 2005; Walch-Liu et al., 2006). Post translational regulation was found in transgenic plants in which dexamethasone was used to activate the ANR1 protein which instigates an increase in lateral root growth, without any effect on primary root growth (Filleur et al., 2005). Similarly, inorganic phosphate uptake can be regulated by the production of transporter proteins, but other mechanisms are involved for post transporter protein production regulation; for phosphorus this could mean the conversion of inorganic phosphate into organic compounds, the reduction of transport through the membrane transporter proteins, and Pi loss by excretion (Schachtman et al., 1998; Bieleski, 1973). In both cases, excess intake of these nutrients has been observed which provides a cost and may lead to decreased fitness (Howitt and Udvardi, 2000).

2.3 Applying optimal foraging theories to plant root foraging behaviour

In the search for an optimal foraging theory it seems reasonable to accept that plant roots foraging the soil for nutrients is analogous to the foraging of an animal, but a discrepancy is that animals consume prey for energy and nutrients, while plants forage for mineral nutrients and water (McNickle et al., 2009). Hence, the currencies for a conceptual framework differ. Plants also forage on multiple sites simultaneously while an animals forages on one site. Despite this, a plant should still be behaving in such a way that benefits its fitness. To make this transition of using animal foraging theories to develop a concept for plant root foraging, McNickle et al. (2009) analysed a set of animal foraging concepts for their applicability to plant foraging behaviour. The modularity of the root system may have parallels with an insect colony, where the fitness benefit is the sum of the performance of every module, but the benefits and costs of nutrient gain and allocation are not directly translated into energy gain.

Root absorption capacity is an important aspect in the plant's economy. It is the absorption rate per unit root and is highly variable among species. Nutrient absorption depends on the nutrient concentration of the soil solution, so the nutrient status of the plant declines when the soil solution concentration declines. Many studies have shown that roots increase their root absorption capacity for the nutrient they have a deficient nutrient status of in the shoots (Chapin, 1980). Absorption rate of one nutrient can also depend on the availability of another nutrient, such as increased nitrogen availability can cause up to a ten-fold increase in phosphorus absorption capacity and vice versa. On the other hand there can be competitive inhibition of ions that share transporter proteins at the root surface. Cation and anion balance is important to maintain uptake of all nutrients, ammonium supply for example can cause imbalance which causes calcium and magnesium uptake to decline (Chapin, 1980). Similarly, Magalhaes and Wilcox (1983) found that ammonium based nitrogen nutrition caused a decline in K, Mg and Ca uptake compared to nitrate based nitrogen, and increased phosphorus uptake. All these processes need to be integrated to understand the dynamics of supply and demand.

The diagram in figure 11 is a summary of the processes and feedback mechanisms reviewed by Chapin (1980). What is interesting is that carbohydrate accumulation is increased when growth decreases, instead of carbohydrates being reallocated to the root system to facilitate root proliferation. This feedback concept is very general and its validity could only be confirmed when the control nutrition is calibrated optimal for the plant species under study. In the field the optimal strategy is dependent on the environment, and since the environment is variable the optimal strategy needs to provide for both short and long term processes (Bloom et al., 1985). The investment currency is also variable and depends on the availability of the different resources and the strength and type of the limiting resource, hence, the environment defines the exchange rates in the system. In the theory Bloom et al. (1985) propose, they assume that that the fitness of a plant is a function of the primary productivity because primary productivity and the quantity and quality of reproductive output is highly correlated (Harper and White, 1974). A plant can maximise it's primary productivity by acquiring the resource when it is abundant, store it, and invest it when the return in terms of growth is maximised, for example when other nutrients are available. Also, since the carbon profit from photosynthesis is the difference between gross photosynthesis and respiration, the plant should stop growing when these two are equal. Primary productivity is maximised when the increase in productivity in response to the change in resource availability is equal for all resources. If this is true, growth is equally limited by all resources.

Because resources can be interchangeable, such as that both phenolics (C) and alkaloids (N) can protect against herbivory, the given balance of resources should equally limit every plant process so in order to maximise primary productivity plants should allocate its resources such that all resources equally limit growth. Hence, plants should invest in shoots when overshadowed by other plants or when nutrient availability is high, both occasions instigate a carbon deficiency. In contrast, nutrient stress causes carbohydrate accumulation in storage and the plant responds by increasing root growth. However, many of the observations that confirm this optimal allocation theory (OPT) could also be explained by the resource allocation strategy in relation to plant size (McCarthy and Enquist, 2007). McCarthy and Enquist (2007) did a study combining the optimal partitioning theory and the allometric partitioning theory which takes the body size into account. They found that much of the variation in allocation patterns can be explained by body size, the remaining variation within a species may partly be explained by the OPT in relation to the environmental factors but to which extent it can be explained is species dependent (McCarthy and Enquist, 2007).

Kobe et al. (2010) showed that biomass allocation to the roots is not a direct measure for investment in nutrient uptake since they found that the largest part of carbon allocation to the roots in nutrient limiting conditions was in the form of total non-structural carbohydrates (TNC) instead of non-storage mass and fine root surface area. When nutrient availability is not sufficient, TNC could be buffered until the nutrient stress is resolved (Kobe et al., 2010). In this study the responses were also species-dependent which makes it likely that life history related to the variability of the environment could explain the buffering capacity of the species. Life history variation is a recurring explanation for the lack of generality of models. The geographical variation in nutrient availability is profound which leads to a range of major adaptive strategies in plants (Grime, 2001).

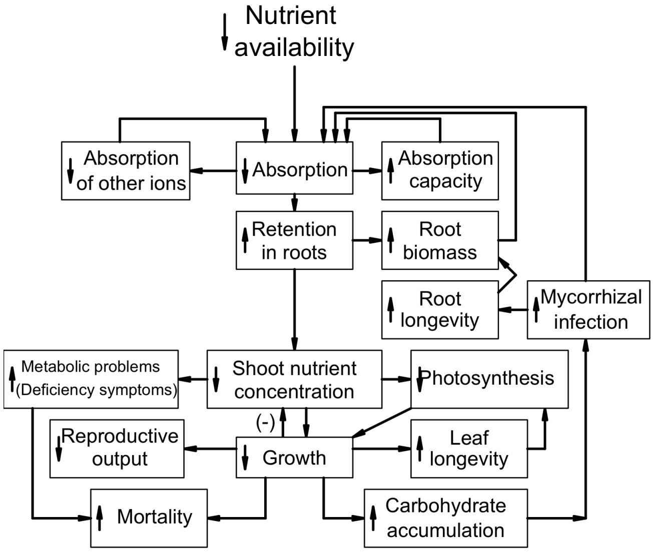


Figure 11: Feedback responses and effect on mortality to a decrease in the availability of a single nutrient. The increase or decrease in a parameter are indicated by the vertical arrows. The arrows between the boxes indicate a positive effect, a negative effect is indicated by (-). Figure and caption reproduced from (Chapin, 1980).

An approach that could overcome this problem of life history and strategy variation is by taking the plant's nutrient demand as the basis for the research, rather than the supply (Mankin and Fynn, 1996). With the assumption that nutrient supply is less important than nutrient demand and nutrient uptake is ultimately driven by demand, Mankin and Fynn (1996) proposed the following relation:

$$U_n = D_n \pm X_n \tag{15}$$

Where U_n is the nutrient uptake in mg per m^2 per hour, D_n is the nutrient demand and X_n is the luxury consumption when positive, and negative when the plant is utilising stored compounds to meet its demand. The size of X_n depends on the particular nutrient, when X_n is constant the plant is at equilibrium. When this value is negative the plant has a demand for the nutrient which should trigger increased uptake. When the plant is in an equilibrium, the demand is determined by the plant's nutrient concentration and the plant's growth rate. Therefore the nutrient uptake can be related to growth while accounting for the plant characteristics, this relation can be described as:

$$U_n = aC_nG_p \tag{16}$$

where,

$a =$ the proportionality constant

$C_{n=}$ the plant nutrient concentration (mg/ g) and

$G_{p=}$ the plant growth rate (g/ m^2/ hr)

Plant growth rate is measured as the increase in dry biomass which is often directly related to the net photosynthetic uptake rate of CO_2. If this relation is linear the growth rate in equation 16 can be substituted by the photosynthetic rate P_n (g/ m^2/ hr) with a proportionality constant b:

$$U_n = bC_nP_n \tag{17}$$

The photosynthetic rate is dependent on photon flux density (PPFD) and therefore we can now relate nutrient uptake to light availability. While there are studies showing an increased uptake of several nutrients upon increased light availability, Magalhaes and Wilcox (1983) found this relation only when nitrate was used and not when ammonium was supplied. This leads to the notion that the model, that describes that uptake is only a result of demand rather than availability, is only valid for mechanisms where nutrient uptake is facilitated, but does not account for unrestricted passive uptake. All the facilitated diffusion and active intake processes selectively transport ions across the cellular membrane so the plant can be selective in the uptake of nutrients that are in high demand.

If we assume that photosynthesis increases proportional to the PPFD that is intercepted by the leaves up to where the plant is light saturated, the photosynthetic rate can be described as a function of the PPFD:

$$P_n = \frac{P_{max}[PPFD]}{K_m + [PPFD} = \frac{U_{max}[PPFD]}{K_m + [PPFD]} \tag{18}$$

where,

P_{max} = the maximum photosynthetic rate (g/ m^2/ hr)

K_m = Michaelis -Menten constant (PPFD at 1/2 P_{max}, or at 1/2 U_{max})

$PPFD$ = Photon flux density (µmol/ m^2/ s)

This is only one example of how nutrient uptake depends on a microclimate parameter. A similar approach could lead to models relating intake to atmospheric carbon concentration, temperature or for example day-length, the key is that this nutrient demand concept explains that the amount and type of every nutrient is determined by plant demand and the demand is determined by microclimate parameters. There are other models of plant photosynthesis and nutrient uptake but they fail to account for variation in demand according to life stage. In this demand model the constants can be easily modified to take life history changes into account.

When using the demand approach to understanding nutrient uptake, the nutrient uptake is regulated on the system level. There are however local responses to nutrient availability at the root tip. Le Bot et al. (1998) reviewed a set of studies which they also combined in a demand framework, specifying the various processes in between the demand, supply, growth and demand feedback loop (figure 12). Groups 1,2 and 3 (figure 12) entail mechanistic approach studies that separate uptake into the actual uptake from soil to root, assimilation and transport, and utilisation for plant metabolism. These are short-time, fine scale studies at the cellular and molecular level.

Most studies have looked at ion transporters and the high and low affinity systems. These studies are difficult to integrate to understand total plant functioning because nutrient solutions are complex ionic environments. It is important to take the nutrient demand into account to understand the feedback regulation of uptake in relation to environmental variables and nutrient accumulation. Groups 4 and 5 skip these successive steps and focus on the ultimate factors that define the equilibrium between demand and uptake and are usually done on monoculture/ field levels on a time scale from weeks to months.

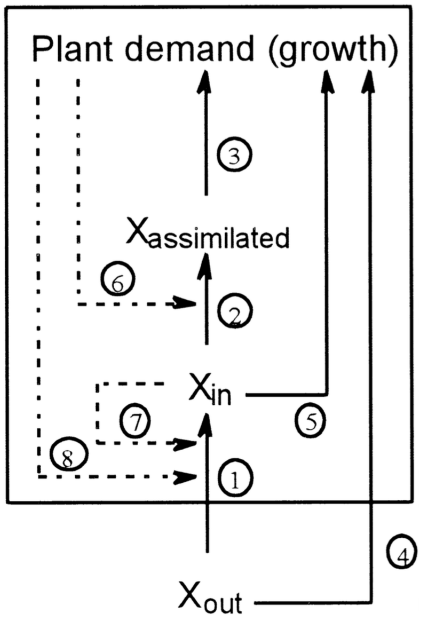


Figure 12: The various approaches to modelling the uptake of a nutrient X. The arrows indicate the target factor and by which explicit factor it is explained. The regulatory processes are indicated by the dashed lines.. Figure and caption reproduced from Le Bot et al. (1998).

2.4 Root architecture

When nutrient uptake is driven by nutrient demand, the uptake regulation takes place on the system level. As mentioned before, also responses on the local level are found which are regulated through different pathways (Forde and Lorenzo, 2012; De Kroon et al., 2009). (Forde and Lorenzo, 2012) reviewed how the availability of nutrients such as NPK and Fe drive root branching, root hair production, root diameter, angle, nodulation and proteoid root formation. They suggested that direct responses to nutrient availability are localised to the root part that is exposed to the nutrient signal. The indirect response is systemic and depends on long distance signals that are given from the shoots (figure 14). They introduced the term trophomophogenesis which describes the change in plant morphology driven by nutrient availability and distribution. An elegant study by Drew (1975) resulted in the clear results on the root proliferation in response to nutrient availability as given in figure 13. The proliferation of roots is strongly decreased in the zones lacking a specific nutrient for all nutrients but potassium compared to the control plant.

In fast growing species adapted to high nutrient availability the most often observed response to nutrient deficiency is a decrease in root-shoot ratio, but this does not look into the root's morphological adaptations. Root branching can be stimulated by nutrient availability (Hacket, 1972; Drew, 1975), which is thought to be a competitive advantage when the nutrient is in a patch and the plant is growing in succession (de Kroon et al., 2012). The strength of this effect is dependent on the nutrient concentration outside the patch, with more branching when background concentrations are low than when these concentrations are high (Forde and Lorenzo, 2012). Root diameter can vary with the environmental conditions, roots hairs provide a means to efficiently explore the soil while having a low maintenance cost. However, fine roots are more vulnerable to desiccation and physical damage than thicker roots, while thicker roots also provide a greater transport capacity. The angle at which roots grow is a measure of gravitropic response of different root types.

Secondary roots grow at an angle from the taproot to grow out of the depletion zone. A response to P was recorded for different types of legumes, in which the basal roots were growing out more horizontally when phosphorus availability was low than when availability was sufficient (Forde and Lorenzo, 2012). However, this response was only observed in 6 out of 16 species and did not occur for any other nutrient deficiencies. Root hairs increase the root surface area and thus the absorptive capacity of roots, and they expand the depletion zone around the root. The root hair density is very responsive to nutrient availability. Morphological adaptations are not only observed in response to immobile nutrients, root hair density is decreased with increasing nitrate availability, while root hair length can be increased (Forde and Lorenzo, 2012).

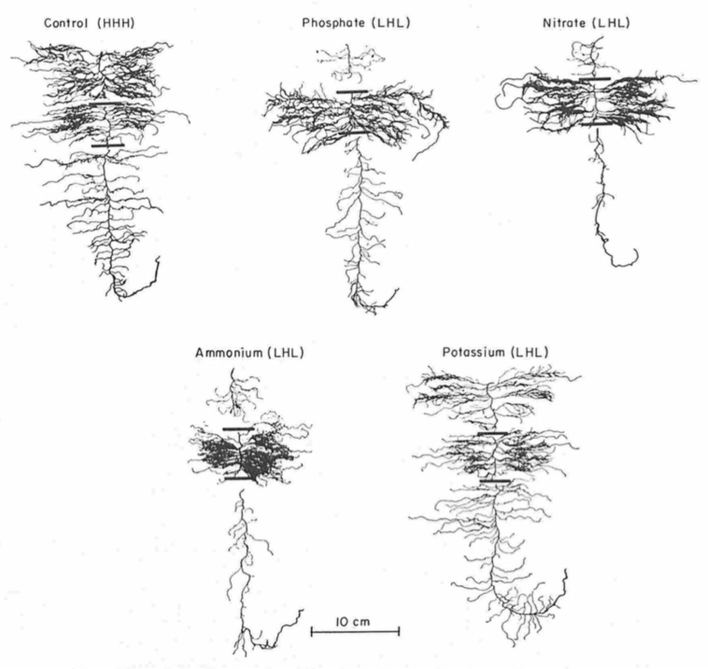


Figure 13: The results of a localised supply of phosphate, nitrate, ammonium, and potassium on root architecture. The control plants (HHH) were given a full nutrient solution to every section of the root system. The roots of the treatments plants (LHL) received a nutrient solution deficient of the specific nutrient to the top and bottom section, and the complete nutrient solution in the middle section of the root system. There are obvious reductions in root proliferation compared to the control plant in the nutrient deficient zones for all nutrients but potassium. Figure and caption reproduced from Drew (1975).

There are two separate systems that regulate nutrient uptake, the first is a direct pathway by signalling at the root tip and responding locally, the second is through the internal nutrient concentration of the plant and it's response on the plant system level (De Kroon et al., 2009; Forde and Lorenzo, 2012). In figure 14a the model for the signalling pathway for the localised response is shown, and the long distance pathway for the plant level response is shown in figure 14b.

The clearest example of a developmental response to localised nutrient supply is the the proliferation of roots within that patch. Jing et al. (2012) found a positive correlation between the ammonium proportion in a patch and the root proliferation in that patch and the nitrogen and phosphorus uptake in *Zea mays* L. However, even though root elongation is regulated at the root tip, the root branching is systemically controlled and depends on the nutrient status of the whole plant and also nitrate availability is found to have both a local and a systemic effect on root branching. Nitrate supply can trigger the transcription of nitrate and nitrite reductase and nitrate transporter proteins. The nitrate reductase (NR) was not essential for the signalling pathway, hence it is nitrate itself that functions as the signal molecule. Even very low nitrate concentrations in the soil solution can be sensed, which was shown with NR-deficient mutants. With these mutants the lateral root elongation in *A. thaliana* was stimulated directly by nitrate, suggesting that regulation is mediated through the sensors and signalling receptors on the plasma membrane, which leads to the distinction between figure 14a and b.

This contradicts the nutrient demand approach of Mankin and Fynn (1996) who explained how nutrient uptake is solely driven by demand and is thus on the systemic level. The localised and systemic responses have to be integrated within the plant, which is only possible if the master regulatory genes in figure 14 are the same so that the signalling pathways converge. This approach allows the localised and system concept to merge and meet the nutrient demand to uptake approach of Mankin and Fynn (1996) on the system level.

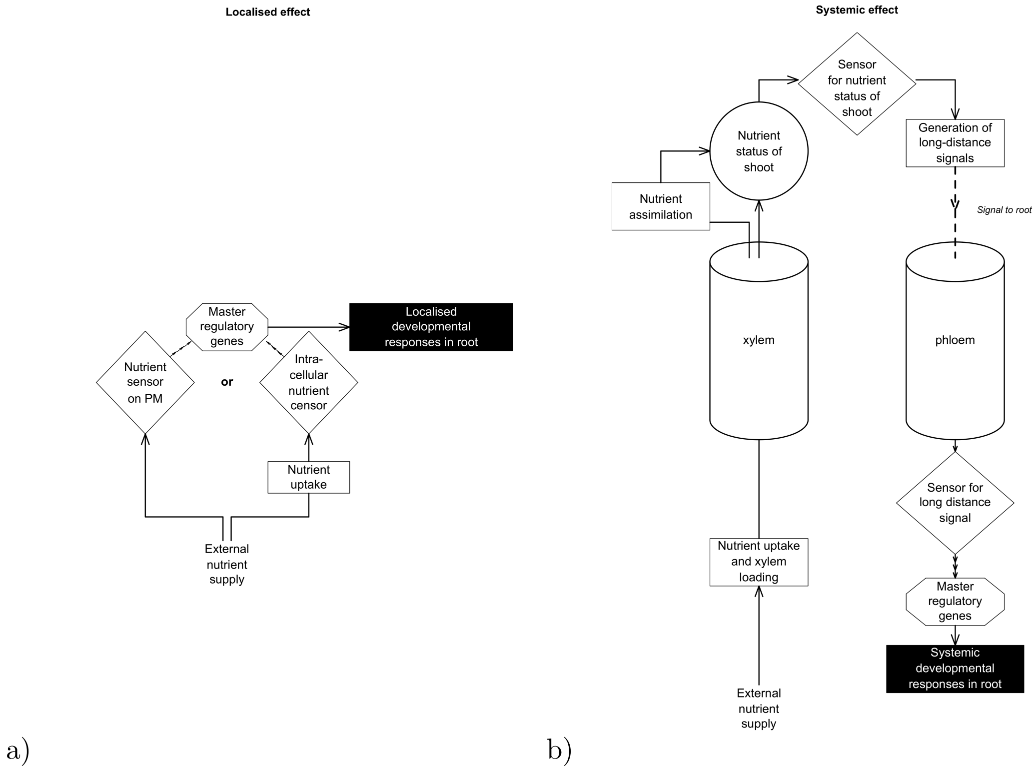


Figure 14: A generalised model for the signalling pathway for a localised response to nutrient supply (a) and for a systemic response to nutrient supply (b). Forde and Lorenzo (2012) propose the existence of master regulatory genes that can regulate the relevant developmental processes. Other environmental signals could also affect these developmental processes which would therefore serve to integrate the plant's responses to various influences. Figures and caption reproduced from Forde and Lorenzo (2012).

Even though root architecture is shaped by elongation, rooting depth, number of laterals and root hair density, major distinctions can be seen between monocots and dicots (Linkohr et al., 2002). Studies that investigate the primary root growth in response to low Pi availability show mixed results, some show an increased or reduced root growth response and others are not responsive (Linkohr et al., 2002). Robinson (1996) showed that the proliferation in response to NO_3^- is similar to the response to P. This is unexpected because the diffusivity of P is low, but rather the diffusivity of nitrate is high and therefore it would be expected that root proliferation is reduced with high nitrate availability. Also because there are disadvantages to selective root placement (Fransen and De Kroon, 2001), especially when the nutrient could travel with bulk flow. One explanation for this proliferation response is that the response is non-specific and that any ionic imbalance may trigger an all or nothing response, where exhaustion of the patch may turn off the proliferation response.

Another reason may be that the availability of one nutrient often coincides with the availability of other nutrients. In competition it would be beneficial to reduce the nutrient availability for the neighbour, while maximising intake for itself (Robinson, 1996). Additionally, there can be a relation between the nitrogen and phosphorus concentration in the shoots and the growth stimulation of locally supplied roots, although it is not consistent for all nutrients. This influence by the shoots can also be explained by the carbon gain related to stomatal conductance and leaf area (Robinson, 1996). For optimality theory it is necessary that the cost currency is limiting which is usually assumed for carbon to be the case. When shoots of locally supplied roots are smaller than control plants and the roots are the same, the root/shoot ratio is higher in locally supplied plants (Robinson, 1996). These roots require relatively more carbon to shoot biomass and the carbon flux to locally supplied roots is higher. This higher carbon flux may sustain and even trigger the proliferation of roots in the supplied regions (Robinson, 1996). However, Thomas (1994) argued that it is possible that carbon is not a limiting nutrient to plants, based on their natural history from the time they were water plants and had a well developed assimilation system in response to low carbon availability. Since the plants moved to land their carbon supply was much higher and therefore land plants should have surfeit carbon availability in relation to the limiting availabilities of nitrogen and phosphorus (Robinson, 1996). Accepting that plants do proliferate roots in such a way that minimises carbon cost and optimises mineral nutrient uptake, there is a key difference between root proliferation into high quality patches with and without depletion (Gleeson and Fry, 1997).

McNickle and Cahill (2009) proposed an explicit framework for the optimal foraging behaviour of plants, and show with an experiment that the marginal value theorem can provide novel insights into root foraging behaviour. Total plant biomass was larger when plants encountered high-quality patches than when they did not. To test the concept of patch leaving time, they grew plants in a rectangular pot with high and low nutrient bands. Patch leaving was considered as the roots continuing growth away from the plant, past the nutrient band. Consistent with the marginal value theorem, the plants spent more time in high quality patches before continuing foraging than in low quality patches. Gleeson and Fry (1997) demonstrated that the plant invested foraging effort in proportion to patch quality. Would there be no depletion, the plant should invest all of its roots only in the one highest quality patch. When the patch quality depletes, which is more likely to be the case in the field than in an experiment, the concept of marginal patch value can be used to understand root proliferation. For the marginal value theorem only one limiting nutrient can be considered and is dependent on the availability in the patch. Every root tip is in a patch and there is no differential transport cost between roots. The total root biomass is constant, and the optimal allocation is such that maximises the sum of uptake of each patch (Gleeson and Fry, 1997). To maximise the total uptake, the root proliferation should be proportional to patch quality. To test this hypothesis, Gleeson and Fry (1997) used a simulation model on *Sorghum vulgare* which showed an increased root proliferation response in high quality patches compared to low quality patches with a really high patch sensitivity. However, these results were only obtained when primary root biomass and experimental error were controlled for because the within individual variation was high. The consequence of patch depletion was addressed by Fransen and De Kroon (2001), who found that root proliferation was beneficial to the young plants, but this advantage disappeared at the end of the growing season when the patches were depleted. This disadvantage may be smaller than the disadvantage of having a competitor growing in that patch instead Fransen and De Kroon (2001). Growing plants in individual pots provides the advantage of studying the responses to nutrients alone, but since plants evolve in successions the mechanisms behind the foraging behaviour may not be understood from these studies.

Competition complicates the understanding of root proliferation mechanisms because different species have different strategies growing in succession. There are several theories explaining the differences in the ability of plants to proliferate roots. One is that root proliferation is adaptive and that it increases nutrient uptake during competition where there is a trade-off between scale and precision (Kembel et al., 2008). It could also be a trade-off where the subdominant species proliferates more to increase its competitive ability, but it might just be a consequence of growth rate Kembel et al. (2008). The trade-off between the scale at which the soil is explored and the precision of proliferation in nutrient rich areas lead to the scale-precision hypothesis of Campbell (1991). This resulted in some discussion about the generality of the concept because there is large variation in the ability of the plant to proliferate roots in nutrient rich soil patches, where some studies find strong evidence for selective root placement (Drew, 1975; Hacket, 1972) and others do not (Hutchings and de Kroon, 1994; Hodge, 2004; Cahill et al., 2010). This variation is explained by factors such as growth speed (Grime et al., 1986) and dominance level when grown in a community (Fransen and De Kroon, 2001). The goal is to understand plant root foraging behaviour and to develop a general concept that applies to most, if not all, plants. Although the strength of the foraging response is often determined by competition and the role of the plant in succession (dominant or subdominant), this variation can be classed in the life-history variation category. From a demand driven approach we need to eliminate the competition effect to understand the basic mechanisms irrespective of the ultimate functional reasons.

Experiments looking at growth or root development responses to nutrient availability are often not comparable, not because the experimental setup may vary slightly, but because the lack of critical consideration of the control group. I believe that a problem comparing these studies and understanding the plant's foraging behaviour, is that the concentrations defined as low versus control, are relative to each species. A plant A that requires twice as much Pi as plant B, might show an inhibited growth response under a regime of concentration x, which is half of the control concentration 2x. Plant B requires less phosphorus because it might be a slower grower, or has a different nutrient requirement due to life history and will not be as limited with a reduction 50% of control concentration. In order to develop a general concept, the control treatment needs to be calibrated on the optimum considering the experimental conditions, around which the high and low nutrient treatments can be defined. Lining up the optima for different species will then allow for comparisons between species.

2.5 *Poa annua*

To develop a general concept, I analysed a range of theories and I derived a set of questions and hypotheses which are laid out in chapter 3. To test these hypotheses, I used *Poa annua* as the model species for several reasons. Firstly, it is monocot and has a fibrous root network, rather than a taproot system. This is a requirement for analysing root investment into multiple nutrient patches. It is also an annual with a short lifecycle which is beneficial for conducting multiple experiments. Even though the aim is to develop a general concept for nutrient foraging, life history needs to be considered when discussing the research findings.

Poa annua is a ruderal and commonly occurs in temperate regions, it is found on most soil types but mostly on moist marshlands that are rich in mineral nutrients. Therefore it is well adapted to moist but it is also relatively drought resistant. It grows best on nutrient rich soils with a fine texture and a pH of 5.5-6. It is dominant in the absence of herbivores and on highly fertile soils but it becomes outcompeted when nutrient availability is low. It is an opportunist when conditions are favourable, it is capable of rapid rehabilitation and seed production after herbivory, but has low seed production in poor habitats. The geographic range in which *Poa annua* occurs has caused some genetic variation between populations (Grime, 2001).

Taxonomically there are two main variants, *Poa annua* spp annua and *Poa annua* spp repens, of which spp annua has an erect growth habit and is quick flowering, spp repens has a (semi) prostrate growth habit and is a short lived perennial with a slower flowering time than spp annua (Tillbottraud et al., 1990; Warwick, 1979). It produces generally 3-8 flowered spikelets and is selfpolinating. Besides seed production, the plant can also spread through tillering.

3 Problem statement and hypotheses

Similar to the approach of Dussutour et al. (2010), plants do not have a central area where nutrient status and availability is processed and intake is regulated. Still, the observations of Drew (1975) have been an inspiration for many to study root proliferation in response to nutrient availability, however, the relation with the organism's state has not been considered. My first question is if plants coordinate the search for multiple nutrients in relation to their nutritional state, to test whether the mathematical concept of what is optimal as described in chapter 1, is applicable to plants. Subsequently, assuming they do, I will make a first contribution to studying how it is regulated. This thesis is about testing the possibility of combining a geometric framework for plants and whether plants defend a target intake of the nutrients under study. In chapter 2 I described the mechanisms of nitrogen and phosphorus uptake by plants because I used these nutrients to test the geometric framework. Nitrogen and phosphorus are both essential nutrients and both the uptake mechanisms and their role in the plant's energy metabolism differ. These differences are necessary to test for the generality of the tested concepts, or may show that their functional needs to be considered in testing a general concept. The first thing necessary to develop a geometric framework, is to establish the optimal intake targets of nitrogen and phosphorus. What is optimal depends on the measure of fitness, for which reproductive success is a straightforward proxy. With different nutrient treatments, I will try to incur situations of over ingestion and under ingestion, and these data should provide a relation between fitness cost and suboptimal nutrition, from which a fitness landscape around the optimum can be derived (Simpson et al., 2004). In this fitness landscape I can show the nutritional rails, however, since the plant can take up the nutrients simultaneously and with different regulatory mechanisms there is no reason to assume the final intake amounts will reflect the ratio of the nutritional rails. If plants indeed regulate, rather than maximise intake, the output should provide the optimal linear nutrition rail from the origin towards the target, which would be line L as described in Houston et al. (2011). With this framework I can test the theory of optimal movement in the nutritional space, which predicts exclusive intake of one nutrient as long as the organism is in a zone that is suboptimal in relation to the optimal nutrient intake ratio. That would be where $\lambda = 0$ or $\lambda = 1$ in equation 12, where $(\lambda(x, y))$ is the diet choice strategy and $\lambda = 0$ or $\lambda = 1$ means exclusive feeding on one nutrient or the other, unless $\triangle(x, y)$ is 0 which is on the optimal feeding rail Houston et al. (2011). There are several ways to study the response to replenishment of the limiting nutrient. The results of Drew (1975) are clear, and there have been various results showing both root proliferation and root growth inhibition for high nutrient concentrations compared to control treatments. I used *Poa annua*, also known as streetgrass or annual bluegrass, as the model plant to construct a geometric framework for nitrogen and phosphorus intake. I tested if it would defend an optimal nutrient intake target and if it would follow the prediction by the theory of Houston et al. (2011), which predicts that the organism forages on one nutrient exclusively when in a nutrient deficient state. If *Poa annua* regulated exclusive feeding, I would test if it did so by investing in root proliferation in the patch with the limiting nutrient, or if other uptake mechanisms such as the expression of transporter proteins or the formation of root hairs are the regulatory response. Unravelling the exact mechanism of uptake is not in the scope of this thesis, rather, carbon investment in the root system should incur a fitness cost, and therefore both root biomass and direct carbon flow into the root system in response to nutrient application of the limiting nutrient is analysed. Hence, there are two aspects of interest, which is first to develop the geometric framework with the fitness landscape, and subsequently to test the optimal movement in the nutritional space. These two aspects are addressed in the following two chapters, for each of which the questions and related hypotheses are stated as follows:

Part 2 Questions:

1. Is there a defined target intake for nitrogen and phosphorus for *Poa annua* and if so, where in the nutrient space is it?
2. Is there an interaction between the intake nutrients on the fitness cost?
3. How do the relations between intake and fitness cost, and any potential interactions shape the fitness landscape?

Part 2 Hypotheses:

1. If plants have evolved to optimise fitness to a defined nutrient intake, there is a cost related to both under and over ingesting that nutrient.

2. Both Betrand's rule and the marginal value theorem predict a fitness relation to nutrient intake that is not linear, hence the fitness landscape will be an either symmetrical or asymmetrical quadratic tilted shape.

Part 3 Questions:

1. Is exclusive feeding observed in any nutrient state that is suboptimal as described by the optimal feeding rail in the model species *Poa annua?*
2. Is nutrient intake regulated by exclusive root proliferation into the resupplied patch of the deficient nutrient?
3. Can the intake response to nutrient supply of the deficient nutrient directly be observed as a carbon allocation response?

Part 3 Hypotheses

1. If nutrient intake selection depends only on the proportion of each nutrient in the available solutions and its effect on state change, and the state change $\neq 0$, the optimal strategy is to maximise or minimise λ, hence, take up one nutrient exclusively.
2. Root proliferation, root hair growth, transporter protein activation and nutrient assimilation require carbon allocation, hence both root proliferation and carbon transport to the root system is exclusively directed towards to patch containing the deficient nutrient.

Part II

Defining the optimal nutrition of ***Poa annua***

4 Experiment 1. Estimating the nutritious range and growth conditions for *Poa annua*

4.1 Introduction

This first experiment provided insight into the overall behaviour of *Poa annua* in terms of temperature, water requirements and most importantly nutrient requirements. In order to build a fitness landscape, three quantities are needed: the intake amounts of the two nutrients under study which are the measure of state and a fitness value as a result of the state. Since plants have several mechanisms to regulate uptake, the different nutrient concentrations only provide variability, and it would be interesting to see if the plant is able to defend a preferred nutrient intake given the environment, and if there is an interaction between the two nutrient availabilities.

I conducted the experiment in fully controlled growth facilities where I estimated the temperature and day-length on the basis of life history and geographical occurrence of *Poa annua*. *Poa annua* is a commonly occurring plant and usually considered a weed by grass producers. The species is an annual which I chose for its short life cycle, which was thought to be from germination to flowering in 6 weeks. Very little research is done on this plant, so knowledge on nitrogen and phosphorus intake preference is lacking. Root proliferation is one of the responses of interest and when creating patches a monocot is preferred over a dicot because the fibrous root network can be split it equal parts without having a taproot in the middle.

The treatments were based on the guidelines of the ammonium type of the Long Ashton solution (Hewitt, 1966), which is an equivalent of the better known Hoagland solutions. I used 5 concentration levels for both phosphorus and nitrogen, with a range from 0 to 1.06 mM of phosphorus concentration and 0 to 0.64 mM of nitrogen concentration. This particular ammonium type solution allows to vary nitrogen and phosphorus independently, although it should be noted that since nitrogen was supplied in the ammonium sulphate-form, the sulphur concentrations also varied between treatments. Additionally, phosphorus was supplied as sodium-phosphate decahydrate, hence the sodium concentrations also varied with treatment. In one study on *Brassica napus,* sulphur did not affect nitrogen uptake or plant development (Abdallah et al., 2010), but Jackson (2000) showed increased yield in relation to sulphur application and Salvagiotti et al. (2009) found that for high nitrogen levels, nitrogen uptake was increased with sulphur availability. This would have been an issue would I have been interested in the fitness response to nutrient availability alone. However, the treatments were merely used to cause variation in the nutrient uptake, after which I would relate fitness to uptake, irrespective of nutrient availability. Similarly, Rubio et al. (2005) found that sodium played a role in counteracting the polarisation of the plasma membrane of root cells due to Pi uptake in *Zosteria marina*, and suggests that phosphorus intake is dependent on a sodium-dependent high-affinity transport system. These are important interactions to consider when translating the research to applications in the field. For finding the optimal intake target they are of lesser importance except that variation in elements other than nitrogen and phosphorus could result in variation in fitness data independent of nitrogen and phosphorus, which would complicate the interpretation of my results.

Even though the reproductive success and nitrogen and phosphorus intake were the ultimate results of interest, I measured the number of leaves and the length of the longest leaf over the course of the experiment and I recorded the time of flowering since germination. This data does relate to the treatment concentrations, rather than to nutrient intake. Both a deficiency in nitrogen (Ma et al., 1997) and phosphorus (Rossiter, 1978) has showed to delay flowering time, Nord and Lynch (2008) found similar results in *Arabidopsis thaliana*, and suggested the delay could lead to increased phosphorus uptake and acquisition before flowering and could therefore reduce the fitness cost due to reduced seed number or quality, but it could also be a result of slower leaf emergence (Ma et al., 1997).

In this chapter I analyse the various responses of *Poa annua* to five different levels of phosphorus and five levels of nitrogen as adjusted from the Long Ashton solution (Hewitt, 1966). I tried to define the intake target for both nutrients and test for any interactions.

4.2 Methods[1]

Germination and growth conditions The *Poa annua* seeds that I used were obtained from Barenbrug Holland B.V. I sowed the seeds on a 50/50 sand-vermiculite mixed substrate. After 11 days enough seedlings had emerged to plant 250 plants in 9 cm ø pots, again on a 50/50 sand-vermiculite mixed substrate. The plants were kept in the Sir David Read Controlled Environment Facility of the University of Sheffield, where the climate in the growth chamber was set to 60% humidity, ambient CO_2 concentration, a day-length of 10 hours with 15 $^{\text{o}}$C. at night and 18$^{\text{o}}$C. in the day. Treatment application started once all seedlings were potted. Photo's accompanying these methods can be found in Appendix A.1.

Treatment preparation The treatments were based on the Long Ashton solution recipe (Hewitt, 1966). The recipe is of the ammonium version with a background solution (table 1 and 2) which contains no nitrate in the solution nor ammonium in any other form than ammonium-sulphate, hence I could control the exact amount of nitrogen by varying the ammonium concentrations (table 3). Phosphorus was added to the solution as $Na_2HPO_4 \bullet {}_{12}H_2O$ (table 4). In table 1 and 2 the concentrations of compounds of the background solution are given which is the equivalent of a 50% strength Long Ashton solution. I used 5 nitrogen and 5 phosphorus concentrations, chosen around the standard 50%, in a full factorial design. I adjusted the pH variation in the solutions to pH=5.5 with diluted HCl.

Of every treatment I applied 25 ml twice weekly until the plant had started flowering, and watering was done every other day to around 80% substrate water capacity. Every plant was randomly linked to a treatment and also the positions in the growth chamber were randomised.

Table 1: Macronutrient concentration of the treatmentbackground solution

Macronutrient	µmM	Long Ashton equivalent
$MgSO_4 \bullet {}_7H_2O$	0.747	50%
K_2SO_4	0.999	50%
$CaCl_2 \bullet {}_2H_2O$	2.000	50%
FeNaEDTA	0.0456	50%

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[1] This experiment was facilitated by the Senior Research Technician and Lab Manager Ms. I. Johnson

Table 2: Micronutrient concentration of the treatmentbackground solution

Micronutrient	µM	Long Ashton equivalent
$MnSO_4 \bullet {}_4 H_2O$	4.999	50%
$ZnSO_4 \bullet {}_7H_2O$	0.504	50%
$CuSO_4 \bullet {}_5H_2O$	0.500	50%
H_3BO_3	25.068	50%
$NaMoO_4 \bullet {}_2H_2O$	0.269	50%
NaCl	50.049	50%

Table 3: Nitrogen treatment concentrations

$(NH_4)_2SO_4$ mM	N mM	Long Ashton equivalent
0.0	0.0	0%
0.802	1.604	20%
1.605	3.209	40%
2.407	4.813	60%
3.209	6.417	80%

Table 4: Phosphorus treatment concentrations

$Na_2HPO_4 \bullet {}_{12}H_2O$	Long Ashton equivalent
0.0	0%
0.266	20%
0.532	40%
0.797	60%
1.063	80%

Seed Collection When flower heads appear from the panicle, the flowers and the pollen become visible. Even though the plant is self pollinating, I helped pollination by spreading the pollen with a brush. Once seeds start to develop, they first appeared green and immature, and they change into orange when maturing. The top seeds mature first followed in sequence down the panicle. Seeds may articulate before they have fully changed colour, therefore the seed bags were placed just before that stage. From 11 weeks after germination, the first plants started flowering. Once seeds started maturing and disarticulating, I placed a glassine bag (Lawson 117) over the flower stalk to collect the seeds. From the moment of seed collection I stopped treatment application. I terminated the experiment when 80% of the plants had disarticulated their seeds, some plants had not set seed at this time. I kept the fresh seeds in a weighing boat covered with pinched aluminium foil. These were kept in an envelope containing silicate gel and stored in the fridge at 5ºC. until all plants had articulated their seeds.

Seed viability From each plant, 20 seeds were surface sterilised in a 71.4g/l Calcium Hypochlorite solution, and whirly mixed for 4 minutes. They were rinsed with sterilised distilled water and plated out on ø9 cm petri-dishes filled with sterilised sand. The seeds were left for two weeks in a 25º C. climate cabinet to germinate.

From three plants per treatment, 100 seeds were weighed to calculate average seed weight (equation 19). Viability can be calculated by multiplying average of one seed in a treatment with the total seed weight per plant in that treatment (equation 20). The germination percentage is derived for every plant from the number of seeds that germinate per 20 sown seeds. Fitness is then given in number of viable seeds per plant (equation 21).

$W300$ =Weight of 300 seeds

$W1$ = Average weight of 1 seed (calculated per treatment)

TSW =Total seed weight per plant

X = Number of seeds per plant

GP = Germination percentage

$Fitness$ =number of viable seeds

$$W1 = W300/300 \tag{19}$$

$$X = W1 * TSW \tag{20}$$

$$Fitness = X * GP \tag{21}$$

Biomass weight The fresh seeds were weighed, one half was stored fresh for the germination experiment and the other half was dried and weighed. I measured plant height up until 11 weeks of treatment initiation and leaf number for 8 weeks. At the end of the experiment I separated the shoots and roots and weighed the fresh shoots after which they were dried at 70ºC overnight. I washed the roots to separate the roots from the substrate, these were also dried. Subsequently dry root and shoot biomass was taken.

Colorimetric determination of phosphorus (adapted from Murphy & Riley, 1962; John, 1970; Leake, 1988). Phosphorus content was measured from seeds, roots and shoots with a Kjeldahl digest method. First, all glassware - tubes and cold-fingers - was washed in 1% HCl, rinsed seven times in distilled water and dried for one day at 80ºC. I ground the dried seed samples in a PM100 Planetary Ball Mill. I cut the root and shoot samples by hand in 1 mm pieces to homogenise the material.

Per sample I digested 0.05 g dry biomass with 1 ml 95% sulphuric acid and I used a mix of $LiSO_4$ and $C4SO_4$ (1:10) as a catalyst. These tubes were heated up to 365ºC and left to digest for 6 hours or when the sample was fully transparent. I diluted the digested samples with dH_2O up to 50 ml, after which I stored them at 5 ºC.

For every sample I made a 4 ml cuvette up with 0.5 ml diluted sample, 0.5 ml ammonium molybdate reagent, 0.2 ml L-ascorbic acid (0.1M) and 2.6ml dH2O and developed for 45 minutes. Before measuring the samples I made a standard curve (fitted r-square > 0.98) with 10 known amounts of sodium dihydrogen orthophosphate (10 mg P L^{-1}). The P-standard and sample's optical density were measured at 882 nm on a spectrophotometer. Protocols for making up the ammonium molybdate reagent, ascorbic acid and sodium dihydrogen orthophosphate were provided by the lab technician Irene Johnson.

Statistical analyses I did all the analyses and plot building in R 3.2.2 GUI 1.66. Contour plots were created with the filled.contour() function of the graphics{} package. Integration of the colour map between the data points is done as default by R. The biomass data were not normally distributed, so group-wise comparisons were always done with the Wilcoxon rank sum test with the wilcox.test() function. I analysed the overall relation between response variables and nitrogen and phosphorus treatments with the non-parametric test Generalised Additive model, with the gam() function in the mgcv package. A generalised additive model (GAM) is based on the generalised linear model (GLM), but the linear predictor is given by a set of smooth functions of the covariates and a parametric component of the linear predictor Wood (2016, 2004, 2011).

The generalised additive models that I used to relate the total plant biomass and the phosphorus intake to the nutrient availability was:

```
gam(formula = TPB ~ P + N + P * N, family=gaussian)
```

and

```
gam(formula = PI ~ P + N + P * N, family=gaussian)
```

Where TPB is the total plant biomass, PI is phosphorus intake, and P and N are the phosphorus and nitrogen concentrations respectively. The data were not normally distributed because the relation is with two independent variables that both cause very low values of plant biomass. Therefore the histogram showed a high occurrence of plants with 0-1 g biomass, whereas beyond these values the data were normally distributed, hence, I used the gaussian family in the model. I did the linear regression for plant biomass in relation to nitrogen for treatment concentrations $\neq 0$ with lm(). The correlation between total plant biomass and phosphorus intake is a Spearman's rank correlation returning a value rho which is between -1 and 1 for perfect correlation, done with the for.test(, method="spearman") function. The linear correlation line was produced with the lm() function.

4.3 Results

Biomass data In figure 15, I plotted the total plant biomass in response to nitrogen (a) and phosphorus (b) treatment. The two plots are essentially the same, on the left the effect of nitrogen is better observed, and in the right plot the response to phosphorus availability becomes apparent.

Table 5: Total plant biomass statistics

Relation to nitrogen availability

N mM	0.0		1.604		3.209		4.813		6.417
p-value		<0.001*				<0.001*			
				<0.001*				<0.001*	

Wilcox rank sum test

*significant increase

Table 6: Total plant biomass statistics

Relation to phosphorus availability

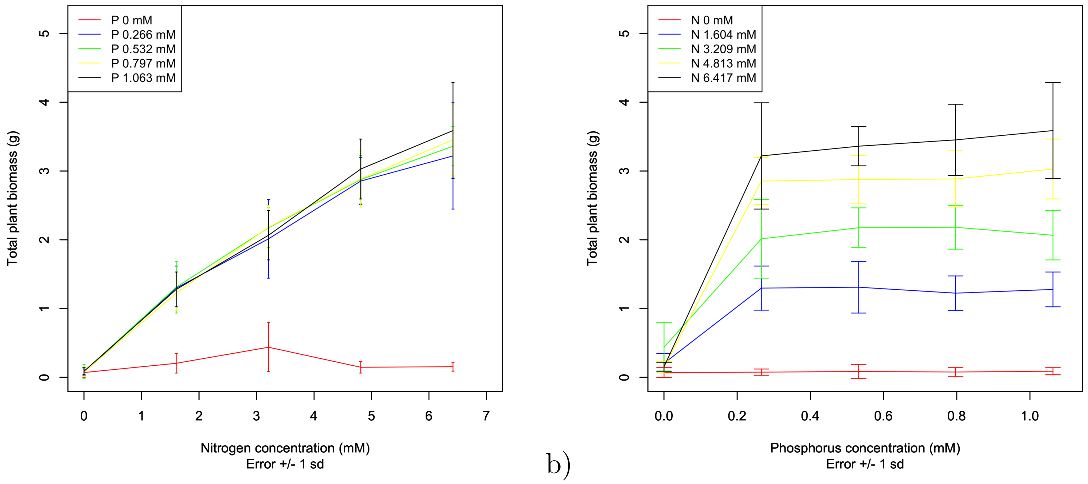
P mM	0.0		0.266		0.532		0.797		1.063
p-value		<0.001*				0.8187			
				0.7777				0.7626	

Wilcox rank sum test

*significant increase

What we see is that there is a large increase between first two phosphorus treatments and no further effect of phosphorus (table 6). On the other hand, total plant biomass increases linearly with nitrogen for all phosphorus treatments other than 0 (table 5). This is important because that means there is no maximum reached for plant biomass production within the range of treatment concentrations that I used.

The generalised additive model to relate total plant biomass to nutrient concentration showed that there was an effect of nitrogen alone and an interaction between nitrogen and phosphorus, but no significant effect of phosphorus alone (table 7). The responses of dry shoot biomass production and root growth are shown in contour plots in figure 16. Shoot biomass and root biomass both increase between 0 and non-zero treatments. Root biomass tends to decrease slightly around 6 mM of nitrogen concentrations, although this effect is not significant. Similarly, the decrease between 0.8 and 1 mM phosphorus is not significant.



a) b)

Figure 15: (a) Total plant biomass in response to nitrogen availability with different lines for the various phosphorus treatments. (b). Total plant biomass in response to phosphorus availability with different lines for the nutrients treatments. Total dry plant biomass increases with increasing nitrogen availability irrespective of phosphorus availability unless phosphorus availability is 0.

Table 7: Generalised additive model for total plant biomass as a function of nitrogen and phosphorus availability.

Coefficients	p-value
P	0.1336
N	<0.001*
N*P	<0.001*
R.squared (adj.)	0.791
Deviance explained	79.3%

* significant effect

Unfortunately the germination test produced no results, almost no seeds germinated and moulds were becoming increasingly visible over time. This indicates that the seeds, which were stored without drying, were too moist and stored in a too humid environment. Therefore I could not calculate a proper fitness measure so I will only further analyse the seed weight data and flowering time. Figure 17 shows the seed weight and this follows a very similar pattern as the shoot biomass and total plant biomass. The time of flowering in figure 17b shows severe nitrogen and phosphorus limitation caused delay of flowering time, or no flowering occurred at all (18 weeks value).

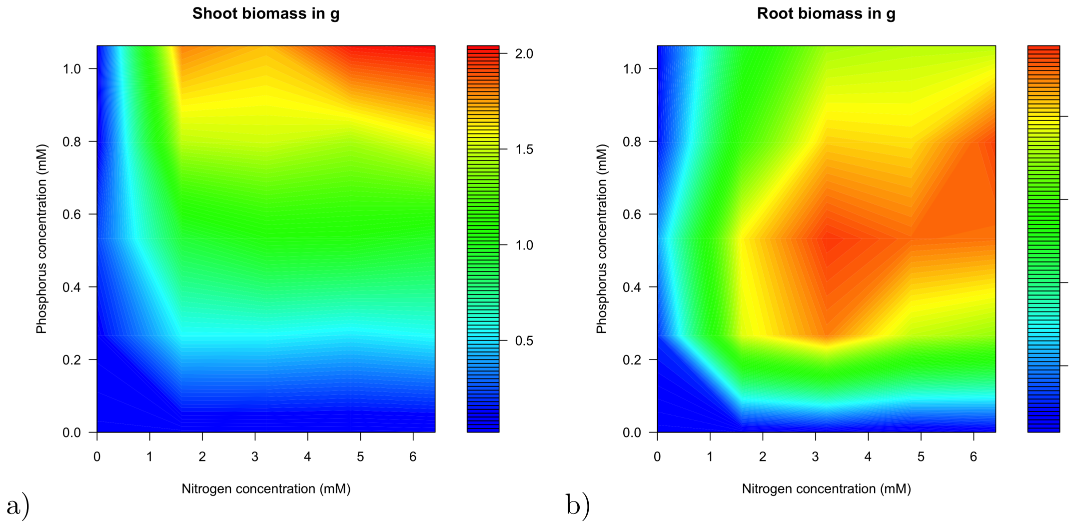
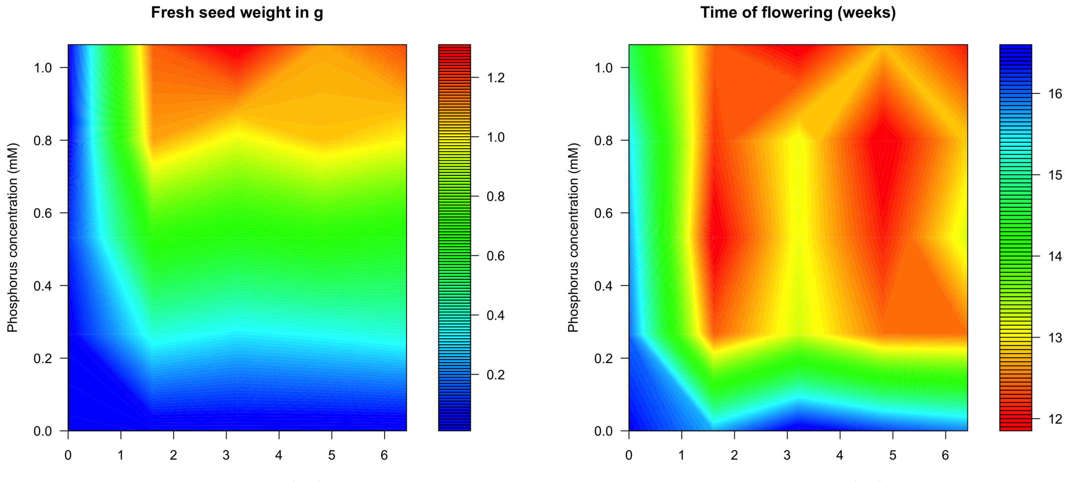


Figure 16: a) Shoot biomass ranging from 0 g (dark blue) to 2 g (red) and b) root biomass ranging from 0 g (dark blue) to 0.5 g (red) in response to nitrogen and phosphorus availability. a) Dry shoot weight is minimal in response to both nitrogen and phosphorus deficient soil solutions. For nitrogen availability >1.5 mM, shoot weight increases with phosphorus availability. b) Root biomass however shows a very distinct pattern, showing reduced root growth in response to sufficient phosphorus availability and reduced root growth in extreme limiting situations. High nitrogen availability has a less profound root growth reducing effect.



a) b)

Figure 17: a) Total fresh seed weight produced per plant ranging from 0 g (dark blue) to 1.3 g (red) and b) flowering time ranging from 12 (red) to 16 days (light blue) in response to nitrogen and phosphorus availability. Seeds were not dried because they were needed for the germination experiment. Note that the heat colours for flowering time are reversed, where early flowering (red) is favoured over delayed flowering (light blue) or no flowering (dark blue). The seed weight follows a similar pattern to the shoot biomass. Flowering time is generally at 12 to 13 weeks after germination, very low phosphorus or nitrogen availability causes a delay in flowering time.

Phosphorus intake In figure 18, the responses to nitrogen (left) and phosphorus (right) are plotted. The variation increases with increasing nutrient concentrations. Both nitrogen and phosphorus positively affect phosphorus intake which indicates an interaction. Each increase in nitrogen concentration causes a significant increase in phosphorus intake, except for the highest two treatments (table 8). The same response is observed for phosphorus concentration. With every increase in concentration the phosphorus intake increases, except for the highest two concentrations (table 9).

Table 8: Nitrogen intake statistics

Relation to nitrogen availability

N mM	0.0	1.604	3.209	4.813	6.417
p-value		<0.001*		<0.01*	
			<0.001*		0.8855

Wilcox rank sum test
*significant increase

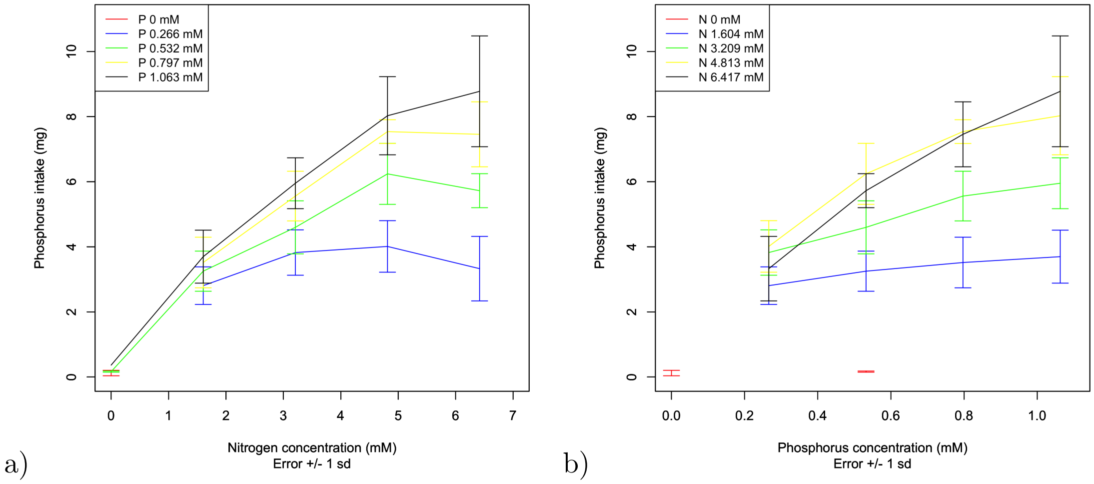


Figure 18: a) Phosphorus intake in response to nitrogen availability. The different lines show the different phosphorus treatments. b) The phosphorus intake in relation to phosphorus availability with different lines for the nitrogen treatments and nitrogen availability. The lowest nitrogen and phosphorus treatments produced insufficient biomass to process and calculate phosphorus content, which results in the incomplete red lines.

Table 9: Phosphorus intake statistics

Relation to phosphorus availability

P mM	0.0	0.266	0.523	0.797	0.1963
p-value		<0.001*		<0.05*	
			<0.001*		0.2474

Wilcox rank sum test

*significant increase

The generalised additive model on phosphorus intake in response to nutrient availability showed a significant effect of both nitrogen and phosphorus availability and an interaction between the two (table 10). That phosphorus intake is affected by phosphorus availability is an interesting finding because it does not affect plant biomass. Therefore I analysed the relation between biomass and phosphorus intake (figure 19). Both variables are dependent so I was looking for a correlation. The plant biomass data are not normally distributed due to a high number of <1 g cases (see methods), and also the P intake data are not normally distributed so I used the Spearman's rank correlation to test for the presence of a relation between the two variable. There is a strong correlation between total plant biomass and phosphorus intake (rho=0.747, $p<0.001$), due to the matching response of biomass and phosphorus intake to nitrogen availability and the interaction effect.

Table 10: Generalised additive model for total plant biomass as a function of nitrogen and phosphorus availability.

Coefficients	p-value
P	<0.001*
N	<0.001*
N*P	<0.001*
R.squared (adj.)	0.785
Deviance explained	78.9%

* significant effect

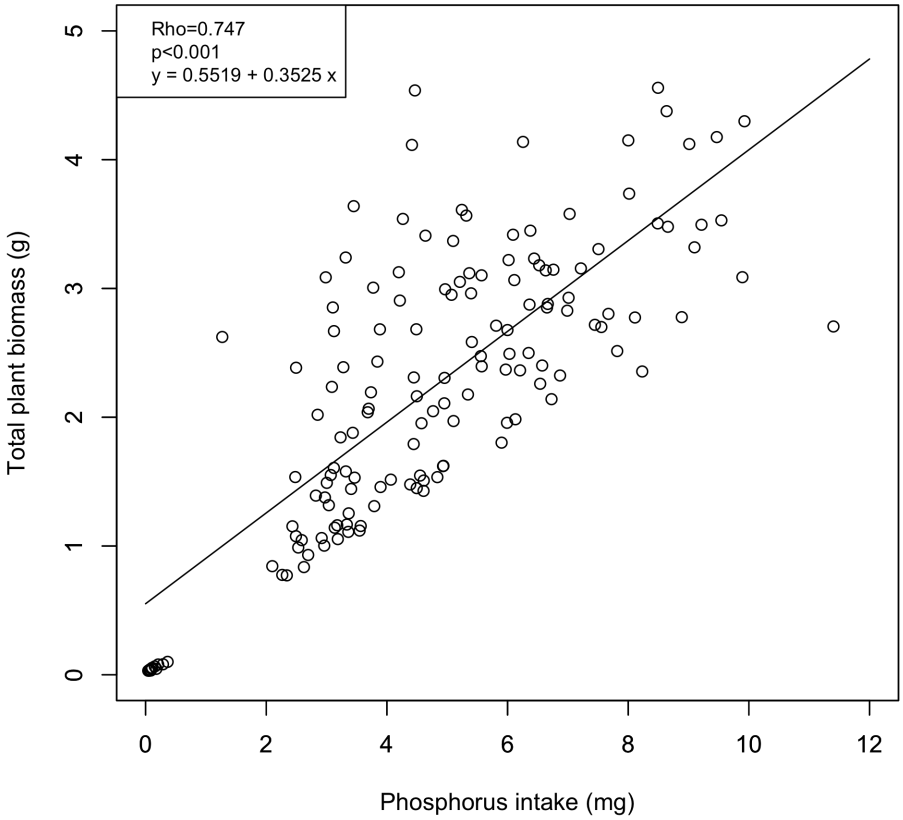


Figure 19: Correlation between phosphorus intake and total plant biomass. It is a Spearman's rank correlation, rho=0.747 which means there is a strong correlation between the two variables. The linear relation is given as y = 0.5519 + 0.3525 x where y= Total plant biomass and x is phosphorus intake, this relation is symmetrical.

4.4 Discussion

The biomass data tell us that there is no maximum reached in the total plant biomass response to the nitrogen treatments that I used. The response to phosphorus was significant between the lowest two levels, which means I need to specify the response within the range of these two values. Because these nutrient ranges need further specifying, I decided not to analyse the nitrogen intake. I could not calculate the fitness data from germination test due to seed deterioration, and therefore I analysed the responses of shoot and root biomass and seed weight to nutrient availability, to establish whether there are any obvious differences.

The shoot biomass shows a similar pattern to the seed biomass, whereas the roots show a slightly different response. The trend in root biomass decline for the highest phosphorus and nitrogen treatments does not result in any significant differences, but could be an interesting observation for the follow up experiments. The flowering response is interesting to form a complete picture of the growth behaviour of *Poa annua.* Medium to high nutrient availability all had a similar flowering time, between 11 and 13 weeks since germination. Below 0.2 mM phosphorus and 1 mM nitrogen concentration the flowering started delaying and for the lowest nutrient treatments the plants would not flower at all. Although these data are not relevant to calculate a measure for fitness, it would indicate a fitness cost were the plants growing in succession. Ma et al. (1997); Rossiter (1978); Nord and Lynch (2008) also found delayed flowering when nutrient availability was low, although the suggestion of Nord and Lynch (2008) that the strategy of delaying is to wait until sufficient nutrients have accumulated to ensure a minimum level of seed quality, does not seem to be a good explanation for my findings. With this theory the seed weight would be expected to be relatively increased with longer flowering time, but the data in figure 17a shows the opposite, seed weight is very low even where the flowering time is delayed (green area figure 17b).

I found a strong relation between phosphorus intake and biomass production. Often an increase in nutrient intake is considered to be a result of biomass increase which could be a reason to analyse plant nutrient concentration instead. However, the plant's biomass is more likely a result of nutrient intake, which could in turn be driven by the balance between nutrient demand and availability (Mankin and Fynn, 1996). From figure 15, observing no change in biomass between 0.266 and 1.063 mM phosphorus, I concluded that the phosphorus concentrations I used were rather high. Although the plants did not show any toxic effects, they no longer increased biomass for higher treatments than 0.266mM P but phosphorus uptake was continued. The explanation for this discrepancy is that both plant biomass and phosphorus intake increased with nitrogen availability, which is a stronger effect than the difference in response to phosphorus.

From these data I can not define the intake target for nitrogen and phosphorus. I can however make a better estimation of where this intake target may be, based on the findings on plant biomass and phosphorus intake. For nitrogen a much larger range of concentrations is needed, whereas for phosphorus I will choose the concentrations between 0 and 0.266 mM, to explore the responses in this area in detail. The question if there is an interaction effect of the two nutrient intake results on fitness cost, can not be answered from these data. However, I conclude there is an interaction effect between nitrogen and phosphorus availability on the production of plant biomass and phosphorus intake. This indicates the uptake mechanisms of nitrogen and phosphorus are interrelated, which confirms the expectations based on chapter 2. What is also important to establish in the next experiment, is if there is a cost related to over ingesting phosphorus. The biomass did not decrease for high levels of phosphorus, and the phosphorus intake did not show a maximum. I observed no toxic effects on the plants applied with the highest phosphorus treatment, so the extension of Betrand's rule to both phosphorus and nitrogen has to be considered in further experiments.

5 Experiment 2. Finding the optimum for nitrogen and phosphorus intake[2]

5.1 Introduction

From Experiment 1 I found within which range of nitrogen and phosphorus application the optimal nutrient intake should be. This point of optimal nutrient intake in relation to reproductive success is needed to construct the geometric framework. To find this intake optimum I did a second experiment with one individual per pot and a range of nitrogen and phosphorus concentrations. I used 6 treatments for both nutrients in a full factorial design giving 36 treatments. The nitrogen concentrations ranged from 0 to 25.67 mM and phosphorus concentrations ranged from 0 to 0.21 mM, with the treatment concentrations increasing logarithmically. Note that the nitrogen range is much larger than in the experiment of chapter 4.

With an improved protocol on collecting and storing the seeds the germination experiment provides the fitness data. These data are calculated as the number of seeds of each plant that would germinate if they had all been sown. With the fitness data in relation to nitrogen and phosphorus intake all the ingredients to combine the geometric framework are available.

A generalised additive model (GAM) can describe the relation between reproductive success and nutrient intake. Be aware that nutrient intake were initially dependent variables of nutrient availability. Now these values for nitrogen and phosphorus intake are taken as independent variables defining reproductive success. The GAM is an extension of the generalised linear model (GLM) by allowing non-linearity and adding smoothing functions. With a GAM, I can estimate the smoothing relationship between reproductive success and phosphorus, and reproductive success and nitrogen simultaneously, and use the sum of these functions to create a link function that relates the expected value to the predictor variables Wood (2016, 2004, 2011). This is useful for my data because the variance is high and even though the optimal nutrient intake ratio should be fixed, giving the slope of the feeding rail from the origin through the intake target (Houston et al., 2011), the observed data may not comply with that hypothesis.

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[2] With major contributions from Dr. D. Childs to the model development

Additionally, with a log-transformation of the nitrogen and phosphorus intake data and a fitted orthogonal linear regression model, the optimal intake ratio can be calculated as described as line L in Houston et al. (2011). For this model, the slope should be equal to 1, as is predicted by the theory (equations 10-13). Plotting this relation in the fitness landscape verifies the location of the optimum that was calculated with the GAM. Ultimately this line separates the exclusive feeding zones ($\lambda = 0 | \lambda = 1$) which is further explored in chapter 6.

In this chapter I present the geometric framework with the fitness landscape of *Poa annua*, with the optimal feeding rail L.

5.2 Methods

The model species, background nutrition and phosphorus determination were as described in Chapter 4. The treatment nutrition for nitrogen and phosphorus concentrations was adjusted. The growth conditions were changed to higher temperatures and longer day-lengths and the seed storage and germination methods were improved compared to Chapter 4. This experiment was done at the Arthur Willis Environment Centre of the University of Sheffield in the greenhouse facility. See Appendix A.2 for photos to accompany these methods.

Growth parameters

Germination and growth conditions I sowed the seeds on a 50/50 sand-vermiculite mixed substrate. After 12 days, the seeds had germinated, I planted 370 seedlings in 12ø pots on the same sand-vermiculite mixed substrate. After two weeks I started applying the nutrient treatments. Throughout the experiment, humidity and CO_2levels were ambient, light was controlled for a 12 hour day length and temperature was 22 degrees C. in the day and 20 degrees C. at night.

Treatment preparation The treatments were based on the Long Ashton solution recipe (Hewitt, 1966). Just like in experiment 1, I used the 50% background solution (table 11 and 12). The six phosphorus concentrations were made up $Na_2HPO_4 \bullet {}_{12}H_2O$ (table 14) and I used $(NH_4)_2SO_4$ for the nitrogen treatments (table 13).The six nitrogen and six phosphorus treatments were combined in a full factorial design, providing 36 different treatments. I corrected for the pH increase, caused by adding $Na_2HPO_4 \bullet {}_{12}H_2O$, to bring back every treatment to pH=5.5 with HCl.

Table 11: Macronutrient concentration of thetreatment background solution

Macronutrient	µmM	Long Ashton equivalent
$MgSO_4 \bullet {}_7H_2O$	0.747	50%
K_2SO_4	0.999	50%
$CaCl_2 \bullet {}_2H_2O$	2.000	50%
FeNaEDTA	0.0456	50%

Table 12: Micronutrient concentration of thetreatment background solution

Micronutrient	µM	Long Ashton equivalent
$MnSO_4 \bullet {}_4\ H_2O$	4.999	50%
$ZnSO_4 \bullet {}_7H_2O$	0.504	50%
$CuSO_4 \bullet {}_5H_2O$	0.500	50%
H_3BO_3	25.068	50%
$NaMoO_4 \bullet {}_2H_2O$	0.269	50%
NaCl	50.049	50%

25 ml of each treatment was applied twice weekly until the plant had started flowering, and watering was done every other day to around 80% substrate water capacity. Every plant was randomly linked to a treatment and also the positions in the greenhouse were randomised.

Table 13: Nitrogen treatment concentrations

$(NH_4)_2SO_4$ mM	N mM	Long Ashton equivalent
0.0	0.0	0%
0.802	1.604	20%
1.605	3.209	40%
3.209	6.418	80%
6.418	12.835	160%
12.835	25.670	320%

Table 14: Phosphorus treatment concentrations

$Na_2HPO_4 \bullet {}_{12}H_2O$ mM	Long Ashton equivalent
0.0	0%
0.013	1%
0.027	2%
0.053	4%
0.106	8%
0.213	16%

Seed handling

From 6 weeks after germination the first plants started flowering, and at 11 weeks the first plants started setting seed. Once seeds started maturing and disarticulating, I placed a glassine bag (Lawson 117) over the flower stalk to collect the seeds. From this moment I stopped giving the treatment solution to this plant, and only continued watering until all the seeds had dropped. I terminated the experiment when 80 % of the plants had dropped their seeds, some treatment concentrations were so low the plant would not set seed at all. I stored the seeds in the fridge at 5^{o}C in boxes with silicate gel to keep the seeds from moulding.

In the first experiment I also collected the seeds. The quality of these seeds quickly degraded due to improper storing. To ensure the seeds quality was maintained at the highest possible level after the second experiment, I put together a seed handling protocol. Following this protocol should ensure that all seeds were handled and stored in the same manner and therefore handling, storage and time between harvests should not affect viability. The seed testing experiment method was based on Willan (1987), Bahadur et al. (2015), ISTA (1996) and Schmidt (2000) and adjusted to this particular experiment. Viability and germination are two terms referring to different types of test result. Seeds deemed viable may not be germinable because of an advanced stage of deterioration or dead tissue in vital parts of the embryo. Also may an immature seed stain normally in tetrazolium staining although it has not achieved germinability yet. Viability tests are often used as a supplement to germination tests in order to examine the character or quality of seeds that have not germinated during the standard test. Vice versa however, a seed that germinated is by definition viable, and therefore I did a germination experiment which should give me an estimation of the number of viable seeds produced per plant. I only collected mature seeds, and therefore I assume a negligible percentage of viable seeds not to germinate. Also, I did not find any significant relation between nitrogen or phosphorus concentrations and age at seeding, therefore deterioration as a result of storage time should not have an effect on the germination results per treatment. This is important because it provides the one dependent variable of the geometric framework which is the measure for reproductive success.

When flower heads appear from the panicle, first the flowers and the pollen will be visible. Even though the plant is self pollinating, the pollination can be helped by carefully spreading the pollen with a brush. Once seeds start to develop, they will first appear green and immature, and they change into orange when maturing. The top seeds mature first followed in sequence down the panicle. Seeds may articulate before they have fully changed colour, therefore the seed bags were placed just before that stage.

To avoid moulds growing on the seeds, the seeds were collected every day and before watering so that the seeds in the glassine bag do not get wet. The seeds were stored in partitioned plastic boxes with a note of the plant number. First these boxes remain open and are placed in the oven at 30ºC for three days, to reduce moisture content. This is the standard drying procedure at Barenbrug Holland B.V. and should bring the moisture content down to about 10%.

After drying, all seeds per plant were combined in the seed boxes. Since the seeds are hygroscopic, seeds were stored in closed boxes with net-balls with silicate gel inside, then placed in a zip bag which was kept at 5ºC.

Measurements and calculations

Seed weight and germination

- Weight per 100 seeds. Since the seeds in these samples are tiny, the dry weight of 100 seeds of each plant was taken.
- ISTA (1996) defines germination as the emergence and development of a tree seedling to a stage where the root system, shoot axis, cotyledons and terminal but indicate whether or not is is able to develop further into a plant under favourable conditions in the soil. These terms are accepted in this experiment for the grass seeds.

In the protocol on tropical seed handling (Schmidt, 2000) data collection is in terms of germinated seed count, so I used 25 seeds of each sample rather than a weight fraction. I had 11 replicates so I used 275 seeds per treatment. This warm germination test reflects the field emergence potential of a seed lot under ideal planting conditions.

Seed viability test Once all the seeds were collected and weighed, I placed 25 seeds of every plant in a grid of 5x5 in a pot with the sand/vermiculite substrate. Every week I recorded how many had germinated, and determined the final germination percentage after three weeks. The remaining seeds were dried over night at 70ºC and weighed.

Plant height and biomass Over the course of 6 weeks, that is up to when the first plant set seed, I measured plant height. Once a plant had dropped all seeds or at termination of the experiment, I separated the roots and shoots, washed the roots, dried everything at 70ºC and weighed the samples.

Nutrient uptake measurements. Nitrogen and phosphorus content was measured from seeds, roots and shoots with a Kjeldahl digest method. First, all glassware - tubes and cold-fingers - was washed in 1% HCl, rinsed seven times in distilled water and dried for one day at 80ºC. I ground all the samples, seeds in a PM100 Planetary Ball Mill, roots and shoots in the Revushchiy Medved ball grinder.

0.05 g per sample was digested with 1 ml 95% sulphuric acid and I used a mix of $LiSO_4$ and $C4SO_4$ (1:10) as a catalyst. These tubes were heated up to 365ºC and left to digest for 6 hours or when the sample was fully transparent. I diluted the digested samples with ultra pure (UP) water up to 50 ml or 20 ml for low weight samples, after which they could be stored in the fridge.

Different colorimetric methods are needed to determine phosphorus and nitrogen concentration in the liquid sample. For phosphorus measurement I used a blue colorimetric analysis (adapted from Murphy & Riley, 1962; John, 1970; Leake, 1988), and for nitrogen a green colorimetric analysis was developed by A. Cotton (personal communication).

Colorimetric determination of phosphorus (adapted from Murphy & Riley, 1962; John, 1970; Leake, 1988). For every sample a 4 ml cuvette was made up with 0.5 ml diluted sample, 0.5 ml ammonium molybdate reagent, 0.2 ml L-ascorbic acid (0.1M) and 2.6ml dH2O and developed for 45 minutes. Before measuring the samples, a standard curve was produced (fitted p-value of 0.98-1) with sodium dihydrogen orthophosphate (10 mg P L^{-1}). The P-standard and sample's optical density were measured at 882 nm on a spectrophotometer.

Colorimetric determination of nitrogen (A. Cotton) 4 ml cuvettes were made up of 0.05 ml sample, 1 ml of Sodium silicate (Solution A), 0.25 ml DIC (Solution B) and 2.5 ml UP water.

Solution A: Sodium salicylate. 20 g tri-sodium citrate, 17 g salicylic acid, 5 g sodium hydroxide and 0.2 g sodium nitro-prusside in approximately 500ml UP water

Solution B: DIC. 5 g sodium hydroxide and 0.4 g dichlorosyonurate in 500 ml UP water.

Before measuring the samples, a standard curve was produced (fitted p-value of 0.98-1) with ammonium chloride (10 mg N L^{-1}). The N-standard and sample's optical density were measured at 650 nm on a spectrophotometer.

Statistical analyses

All the data was analysed in R 3.2.2 (GUI 1.66 Mavericks build). The following packages were used:

- mgcv package - Mixed GAM Computation Vehicle with GCV/AIC/REML Smoothness Estimation, version 1.8-12.
- resample package - Resampling functions, version 0.4.
- onls package - Orthogonal Nonlinear Least-Squares Regression, version 0.1-1.

Defining the optimum Throughout the modelling process I was advised by Dr. D. Childs, who also provided the code for the final model.

I used the gam function of the mgcv package to create a model relating the number of viable seeds to the nitrogen and phosphorus intake including their interaction. The first model was constructed as:

Model 1: gam(Fit ~ te(N, P), family = gaussian, data = data)

Where Fit is the fitness as number of viable seeds, see methods of chapter 4 for calculations. N and P are the total amounts of nitrogen and phosphorus in the plant, in mg. The te() function is a tensor product smoothing function, which is used when the independent variables are on different scales, a potential interaction is also considered with this construction.

To deal with the high number of zero-values, the optimum was found through a combination of two models. Model 2 describes the probability that the fitness is not zero. Model 3, fits a model to the non-zero cases:

```
Model 2: gam(y ~ N * P+ I(N^2) + I(P^2), family = binomial (link = logit),
    data = mutate(data, y = as.integer(Fit>1)))
Model 3: gam(GP ~ N *P + I(N^2) + I(P^2), family = nb(link = "sqrt"), data
    = filter(data, Fit>1))
```

The predicted values can now be derived from simply multiplying the probability that the fitness is not 0, and the predicted values of the model for non-zero observations, which are both produced by predict(Model ...).

The optimisation of the models was done with the following function:

```
optim.fn <- function(par) {
        newdata <- data.frame(N = par["N"], P = par["P"])
        p.not.zero <- predict(Model 2, newdata = newdata, type = "re-
            sponse")
        fitness <- predict(Model 3, newdata = newdata, type = "response")
        p.not.zero * fitness }
```

This function calculates the predicted data by multiplying the predicted response of the probability the data is non-zero and the predicted non-zero data. This is used in the following function to calculate the optima with the optim() function:

```
Model 4: optim(par = c(N = 200, P = 10), fn = optim.fn, method = "L-BFGS-B",
    lower = c(0, 0), upper = c(300, 30), control = list(fnscale = c(-1, -1)))
```

The initial parameters are estimated at a nitrogen intake of 200 mg and phosphorus intake of 10 mg. The L-BFGS-B method is a limited memory modification of the Broyden, Fletcher, Goldfarb and Shanno method (Byrd et al., 1995), which uses the values and gradient to combine the optimisation image and allows for upper and lower bound constraints . This is ideal for the fitness landscape since we are looking for a gradient in all directions from the optimum, and we are looking for the optimum within the nitrogen intake range of 0-300 mg and a phosphorus intake range of 0-30 mg.

Defining line *L* To find line *L* dividing the areas of exclusive feeding, I did an orthogonal regression with the onls package. First I log-transformed the data to make it more interpretable, and because the treatment concentrations increased exponentially and the variability in the data increased with increasing concentrations. The regression in the log-space has to be linear and the slope=1 is fixed for it to be linear in the untransformed space, as required by the theory. Hence:

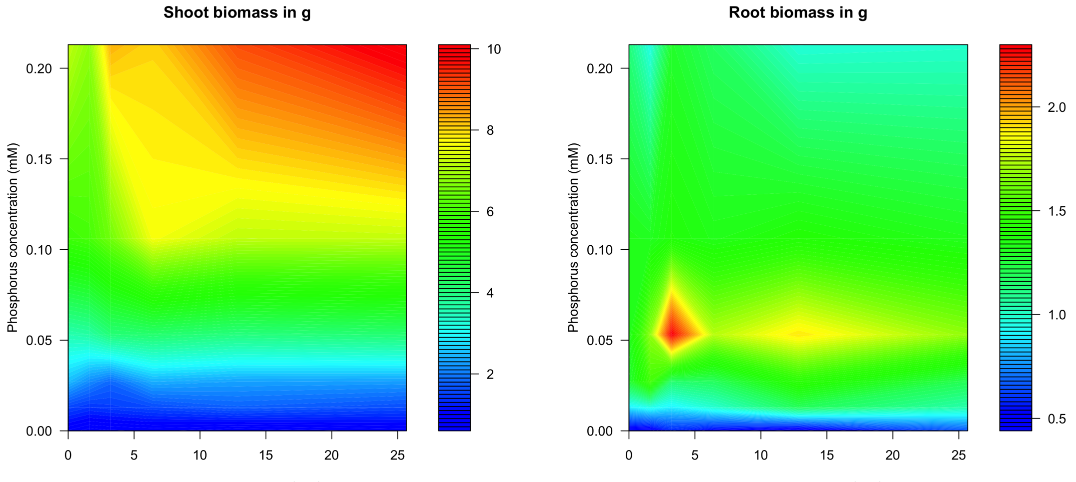
Model 5: onls(log(P) ~ a + log(N), data=data, start=list(a=1), window=120)

P is the phosphorus intake in mg and N is the nitrogen in take in mg, both are log-transformed for the model. The window value is a value for the window around the predictor values in which the optimisation function in the package searches for the minimum distance to the data. The intercept is free and defines the linear function for the untransformed space.

Root biomass model Root investment could be a measure of carbon investment for nutrient uptake and therefore I replicated the steps of model 2, 3 and 4 by substituting Fit by total root biomass.

5.3 Results

Biomass findings To get a first idea of how the plants behave in response to the different treatments, I compared the root biomass, shoot biomass (figure 20 a and b), seed weight and reproductive success (figure 22 a and b). The contour plots show the investment in every plant part in relation to the 36 treatments, of which the axes show the cumulative amount supplied over the course of 6 weeks.



a) b)

Figure 20: The nitrogen and phosphorus treatments were varied with 6 concentration levels. a) Total dry shoot weight ranging from 0 g (dark blue) to 10 g (red) in response to nitrogen and phosphorus availability. b) Total dry root weight, from 0.5 g (dark blue) to 2.5 g (red), in response to nutrient availability.

Root biomass and shoot biomass do not show the same response to nutrient availability. Shoot growth is almost linearly increased by phosphorus availability, except for the lowest nitrogen treatments where the 0 nitrogen treatment inhibits growth. Root biomass follows an interesting pattern, with the highest investment for the second lowest treatment of both nitrogen and phosphorus. Beyond this point increasing nitrogen concentration has no further effect, while phosphorus availability shows a consistently higher root investment at 0.05 mM phosphorus irrespective of nitrogen availability. This is in line with my earlier notion that an increase or decrease with phosphorus availability depends on the reference point. In the treatment with 3.2 mM nitrogen and 0.05 mM phosphorus, both nitrogen and phosphorus availability are low enough enough to instigate a root foraging response, below these levels the nutrient availability is too low to have the resources for root proliferation, which is also reflected by the low shoot biomass in figure 20a. The lack of root biomass variability in response to nitrogen availability may be explained by the fact that the nutrient treatments were supplied directly at the roots, which prevented the formation of a depletion zone and hence root proliferation was not necessary. Regulation for up or downregulation of nitrogen would then occur at the root surface by increasing root surface area (Forde and Lorenzo, 2012) or the activation of transporter proteins (Filleur et al., 2005).

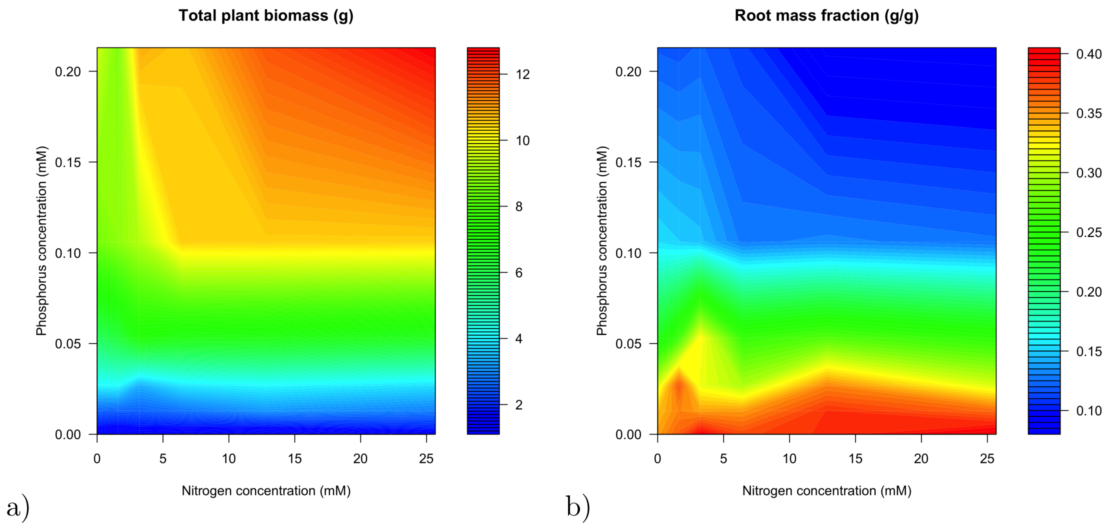


Figure 21: a) The total plant biomass ranging from 1 g (dark blue) to 13 g (red) shows a very similar pattern to the shoot biomass (figure 20a). Both nitrogen and phosphorus increase cause an increase in biomass, where phosphorus limitation has a stronger limiting effect than nitrogen limitation has. b) Root mass fraction (RMF) is the dry root biomass per total dry plant biomass, here ranging from 0.08 g/g (dark blue) to 0.4 g/g (red). The pattern shows a clear decrease in relative root growth for increasing phosphorus availability, and no response to nitrogen availability. The area where relative root growth is highest, total plant biomass (a) is at its minimum.

Figure 21 shows the total plant biomass (a) and the root mass fraction (b), that is the relative root weight to the total plant biomass. The relative root investment is highest when phosphorus is limiting and this limitation is causing very low total plant biomass. The tipping point is around the 0.05 mM phosphorus concentration where absolute root biomass is at its maximum (figure 20b) and nutrient uptake seems to be sufficient to increase total plant biomass. For concentrations higher than 0.05 mM phosphorus root proliferation is no longer necessary to acquire sufficient phosphorus and the root mass fraction decreases (figure 21b).

In figure 22a, the seed weight is plotted in response to the nutrient availabilities. In figure 22b, the germination potential is plotted, so we can see if there are any differences, or if seed weight would be a good measure for fitness. Interestingly, for high phosphorus availability (>0.10 mg), there is a small decline in fitness with increasing nitrogen excess (>12 mg), while this nitrogen availability does not affect the seed weight. In other words, excess nitrogen causes a slight degradation of the seed quality, while phosphorus availability causes an increase in seed quality.

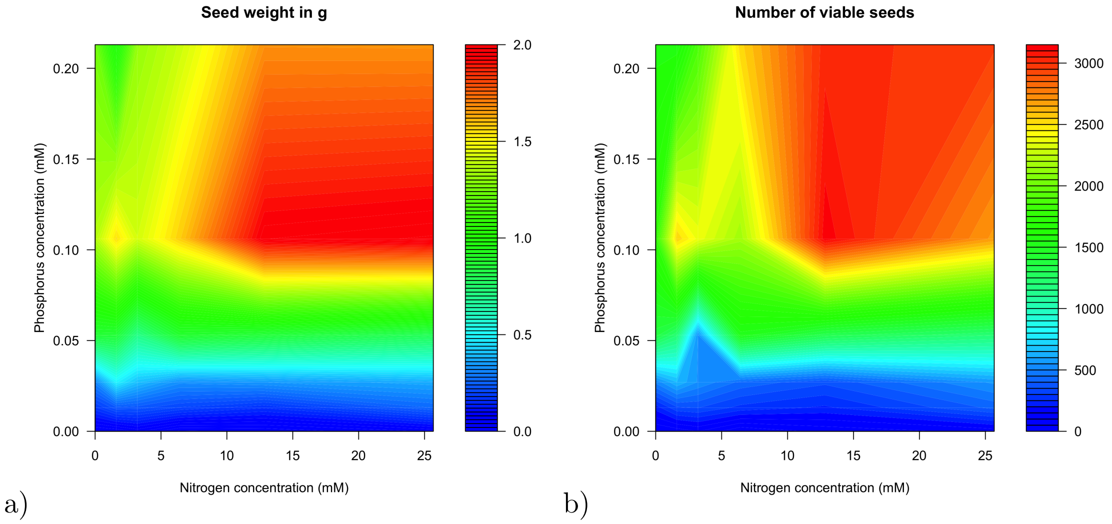
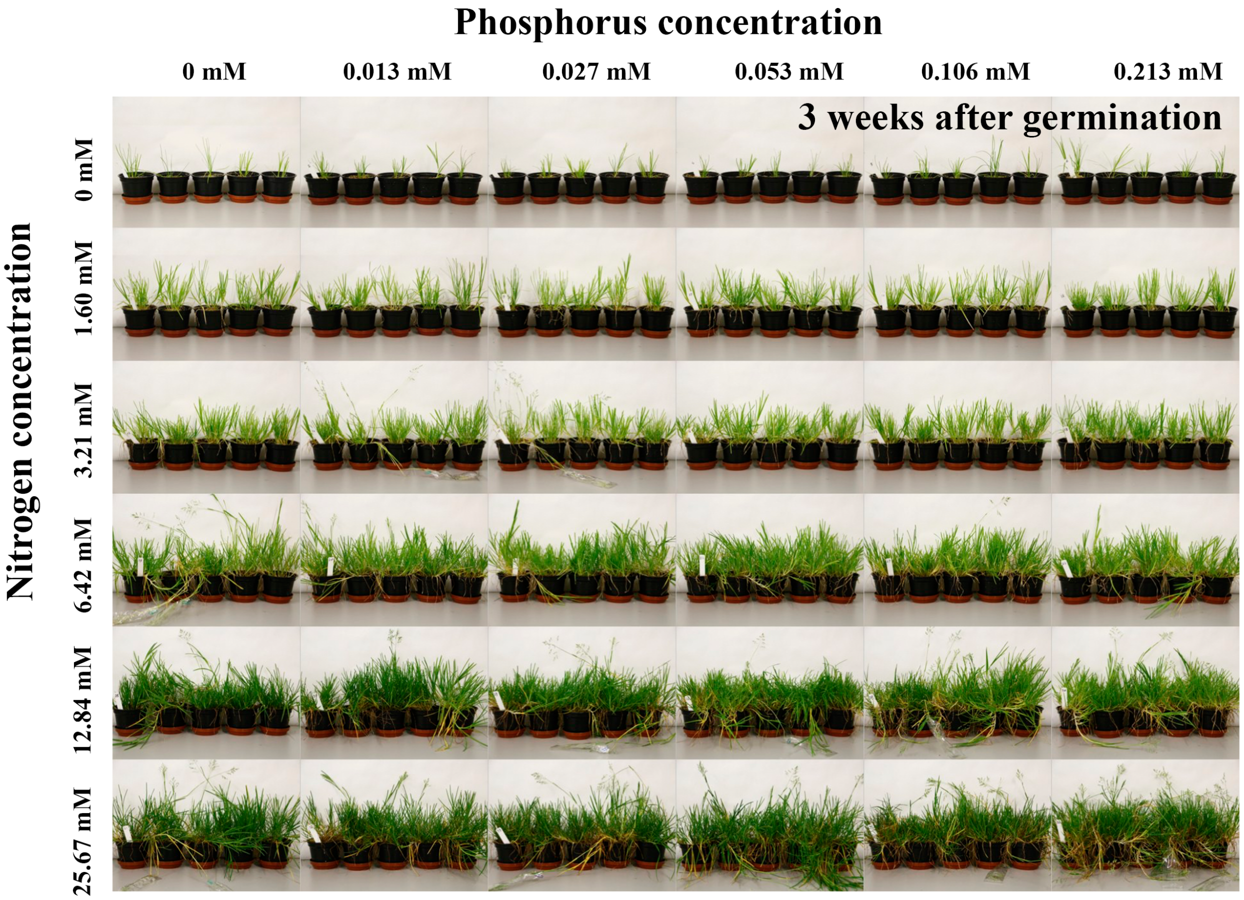
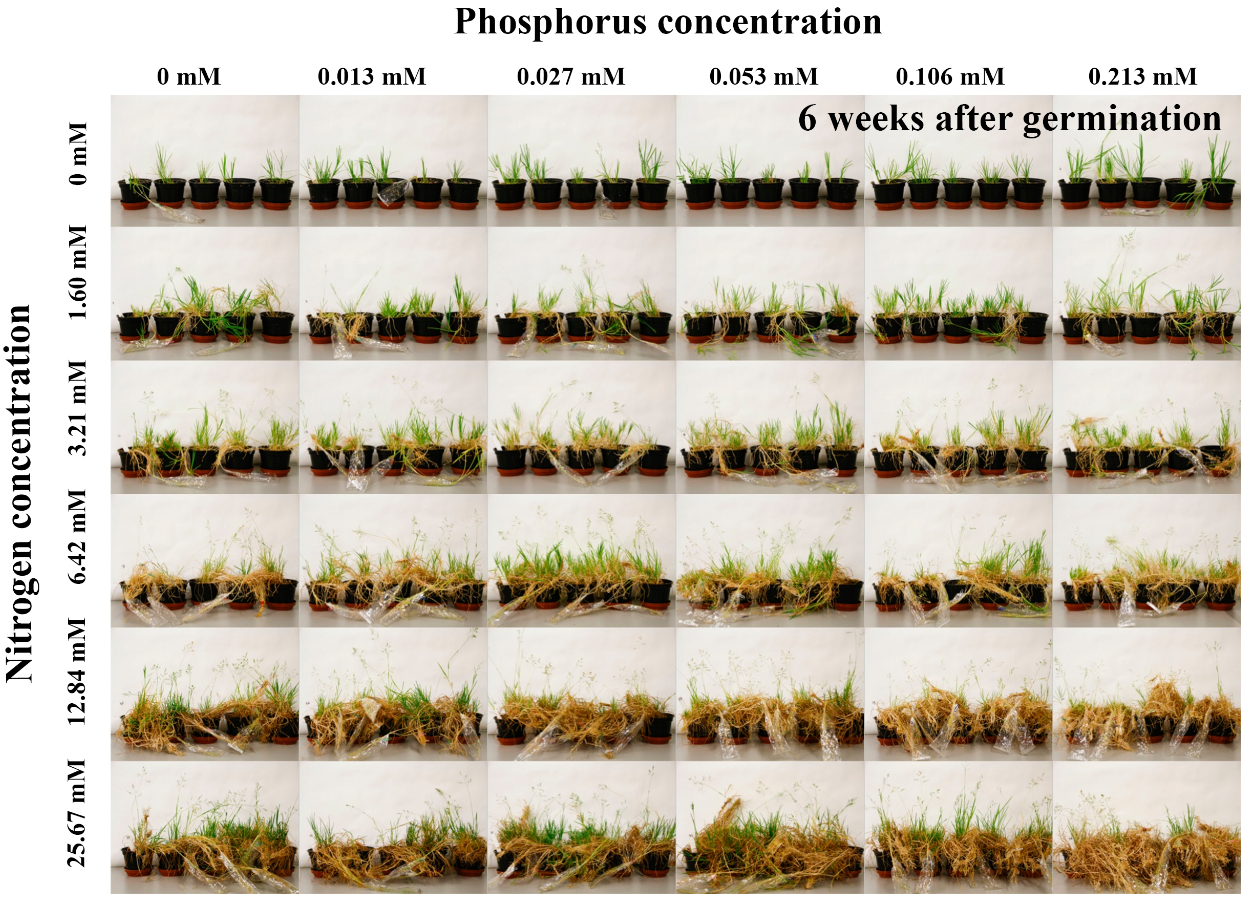


Figure 22: The nitrogen and phosphorus treatments were varied with 6 concentration levels. a) Total seed weight (dehydrated to 10% moisture) ranging from 0 g (dark blue) to 2 g (red) in response to nitrogen and phosphorus availability. b) Fitness as the number of viable seeds, from 0 (dark blue) to 3000 (red), in response to nutrient availability.

The difference between the fitness and seed weight figure (figure 22) is only small, where the highest levels of nitrogen cause a slight decline in number of viable seeds given that phosphorus availability is high, whereas high levels of phosphorus cause a small decrease in seed weight, however, these results only show a trend and do not show a significant decrease in seed numbers for high nutrient availability. The fitness proxy for finding the optimal intake target was calculated as the number of viable seeds.

The intake target can not be derived from these plots because the contour plots relate the data to the treatment levels, rather than the intake levels. Both the root growth response and the shoot growth response to the nutrient availability, in interaction with that nutrient availability, should determine the nutrient intake. The fitness is ultimately determined by nutrient intake, and this leads us to the question of whether there is a defined target intake which relates to maximum fitness.


Phosphorus concentration
0 mM
0.013 mM
0.027 mM
0.053 mM
0.106 mM
0.213 mM
3 weeks after germination
Nitrogen concentration
0 mM
1.60 mM
3.21 mM
6.42 mM
12.84 mM
25.67 mM


Phosphorus concentration
0 mM
0.013 mM
0.027 mM
0.053 mM
0.106 mM
0.213 mM
6 weeks after germination
Nitrogen concentration
0 mM
1.60 mM
3.21 mM
6.42 mM
12.84 mM
25.67 mM

Model 1 results Model 1 provided the basis for the modelling process, from which I produced the plot in figure 23. It shows the predicted nutrient intake and fitness landscape in which the optimum lies within the 3000 boundary, which means the plants in this space would produce 3000 viable seeds in one life cycle. For a better estimation of the mean intake and fitness values, I resampled the data set (1000x), and the modal means of every treatment are plotted with the corresponding 95% confidence intervals in figure 23.

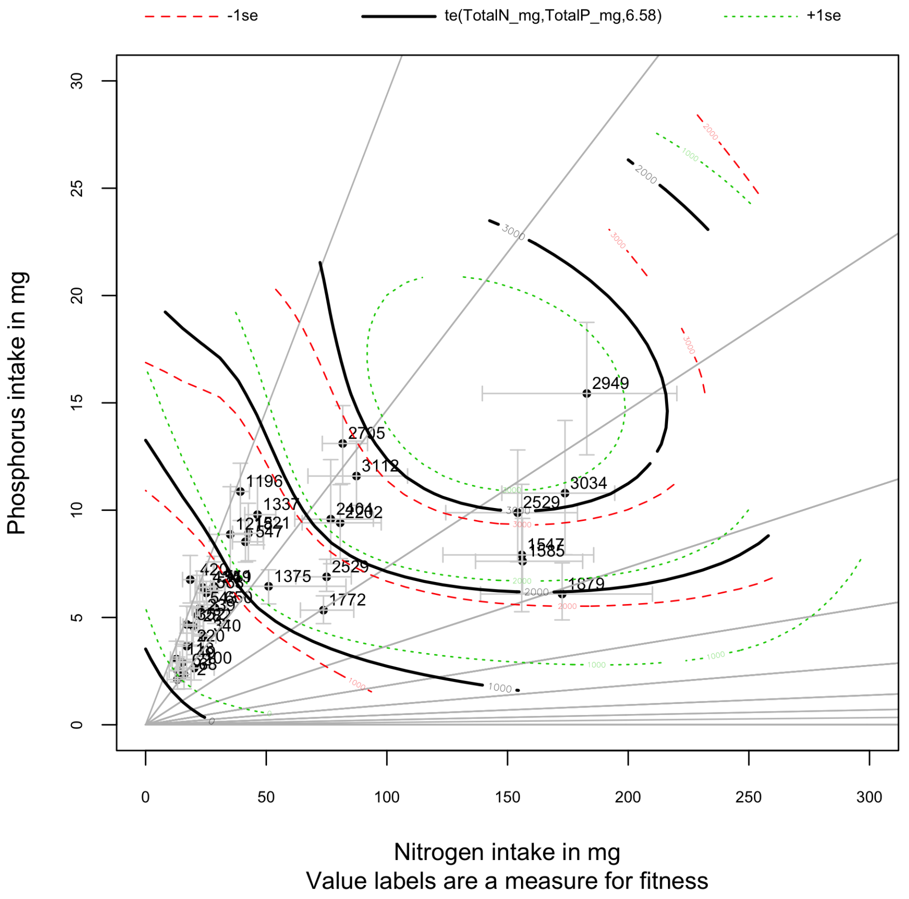


Figure 23: The predicted fitness landscape based on model 1 and the observed data alone. The observed data are the nitrogen and phosphorus intake amounts as measured in the colorimetric determination. The reproductive success, that is number of viable seeds, finds its optimum in the inner thick black ellipse which shows the 3000 boundary. The green dashed lines and the red dashed lines show the upper and lower 955 confidence boundaries of the model estimation. The data points reflect the modal mean for number of viable seeds and nitrogen and phosphorus intake from the 1000x resampled datasets. The grey bars to the data points show the 95% confidence interval for nitrogen and phosphorus intake of the intake means. The diagonal grey lines reflect the nutrient ratio's of the various treatments.

From this plot, it was striking that the intake ratio followed a distinct slope for low nitrogen and phosphorus intake levels, whereas this ratio was not maintained for some treatments that result in a relatively high nitrogen intake. The optimum also matches a lower N/P intake ratio than is seen for lower intake values.

Because the model that I used was based on a gaussian function, there were some doubts about the quality of the model since the data contains a high number of zero values. After running a gam.check(), which runs a simulation based check on the basis dimensions and produces 4 residual plots, this showed that the mean-variance relationship is unacceptable, caused by the presence of many zero values. Therefore we explored other possibilities which led to a combined model of model 2 and model 3.

Model 2 calculates the probability of non-zero's in the data, and subsequently a negative binomial to obtain predicted values for the non-zero cases. With model 3 the fitness values can be predicted for the non-zero values. The overall expected fitness is given by the product of the chance of being non-zero and the predicted fitness.

The geometric framework With the new data, the predicted values from model 2 and model 3, I could plot the fitness landscape as in figure 24. The maximum predicted reproductive success of 3646 viable seeds was found at a nitrogen intake of 127.9 mg and a phosphorus intake of 26.0 mg over the course of 6 weeks (figure 24). The fitness is reduced with surpluses and deficits of both nutrients. The ellipse shows an almost symmetrical quadratic up to the 2500 fitness cost boundary, so in this space the fitness cost of the deficit of either nitrogen or phosphorus is almost equal to the fitness cost of a surplus. The ellipse is tilted which reflects an interaction cost, the cost of excessively ingesting phosphorus is increased with the decrease or increase in nitrogen intake deviating from the optimum, and vice versa.

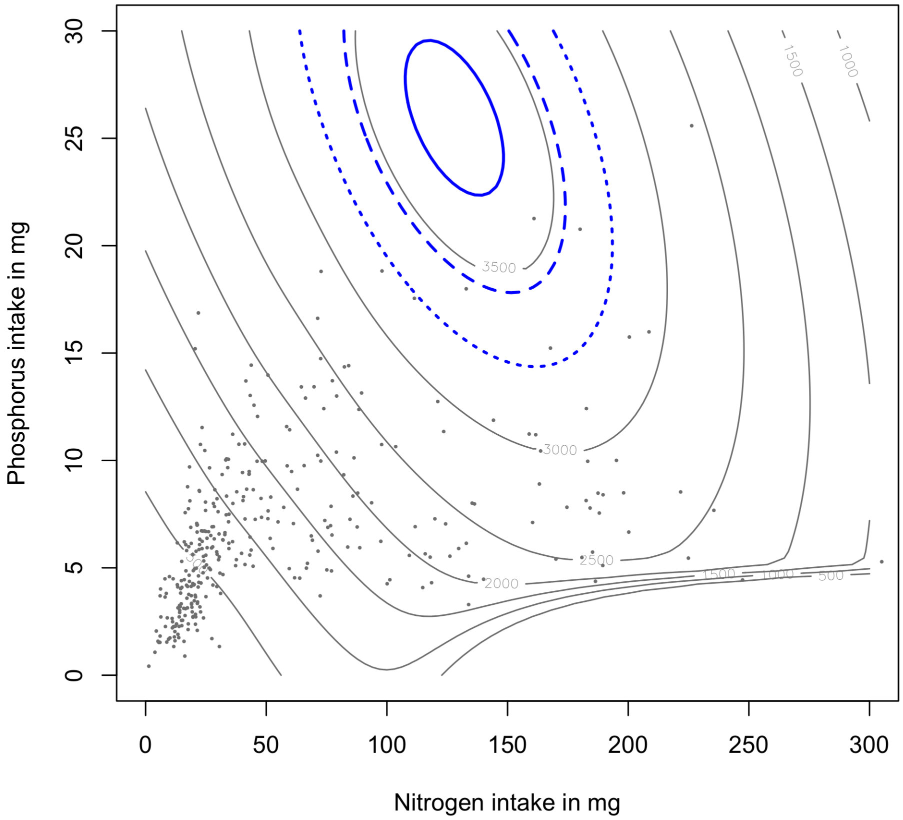


Figure 24: Generalised additive model plot, based on the model predictions for reproductive success. The maximum lies within the space higher than 3500 viable seeds per plant. The solid blue line reflects the 1% of the maximum fitness contour, the semi-dashed contour shows 5% and the dashed line 10% of the maximum fitness set. The grey contour lines show the intake space for 500 seeds increments. .

In addition to the generalised additive model, I did an orthogonal regression to find the line of minimum summed distance to the data with equal weight to both dimensions, the minimum distance is found by minimising the euclidian distance in both axes. I found this regression line for the log-transformed data and then transformed the function for the untransformed space (figure 25). This figure reflects the direct relation between nitrogen and phosphorus intake and represents the optimal feeding rail as proposed by Houston et al. (2011). Given that the optimum intake lies at 127.9 mg N and 26.0 mg P (figure 24), the shortest way for a plant to reach that point is to feed along the line L as described in the theory. The regression line reflects the intake ratio of the nutrients and should therefore be line L going through the optimum.

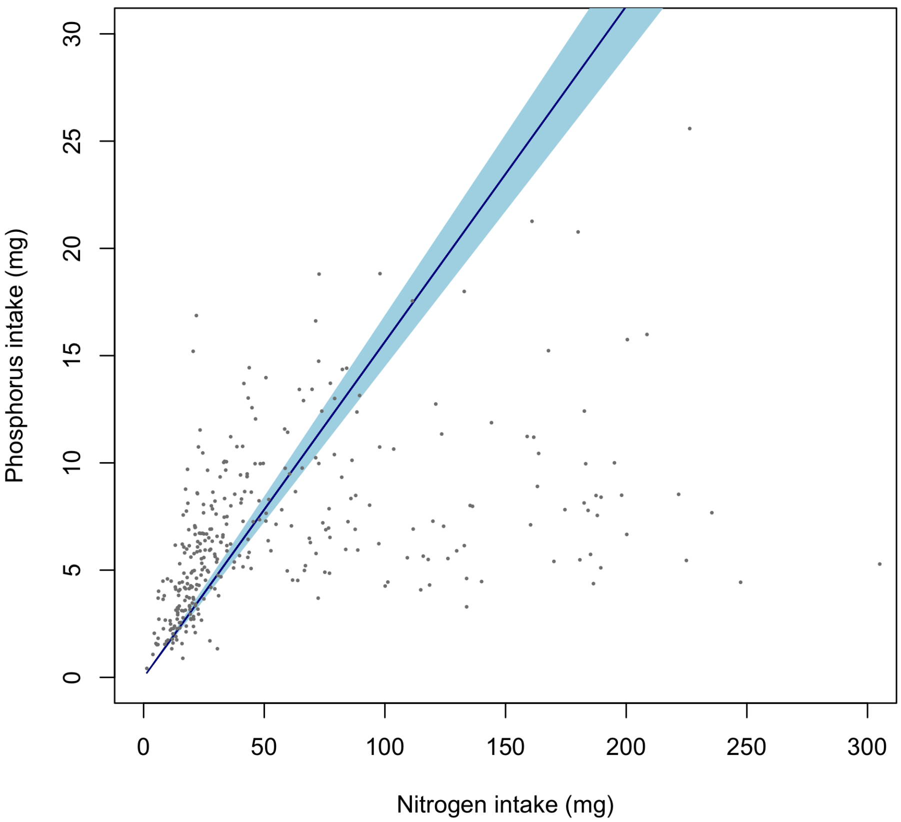


Figure 25: The dark blue linear line is the result of the orthogonal regression model done in the log space and transformed to the linear space for comparison with previous figures, The light-blue shade is the 95% confidence interval for the intercept.

The slope of L was calculated from the orthogonal regression as in model 4, which returned a standard error S= 0.061. This regression model is also a means to verify the optimum intake location of the GAM in figure 24. Figure 24 and 25 are combined in figure 26. The area from the upper limit of the 95% confidence interval to the regression line goes through the space of the highest 1% reproductive success, and the full confidence interval is within the 5% highest predicted values for reproductive success (number of viable seeds).

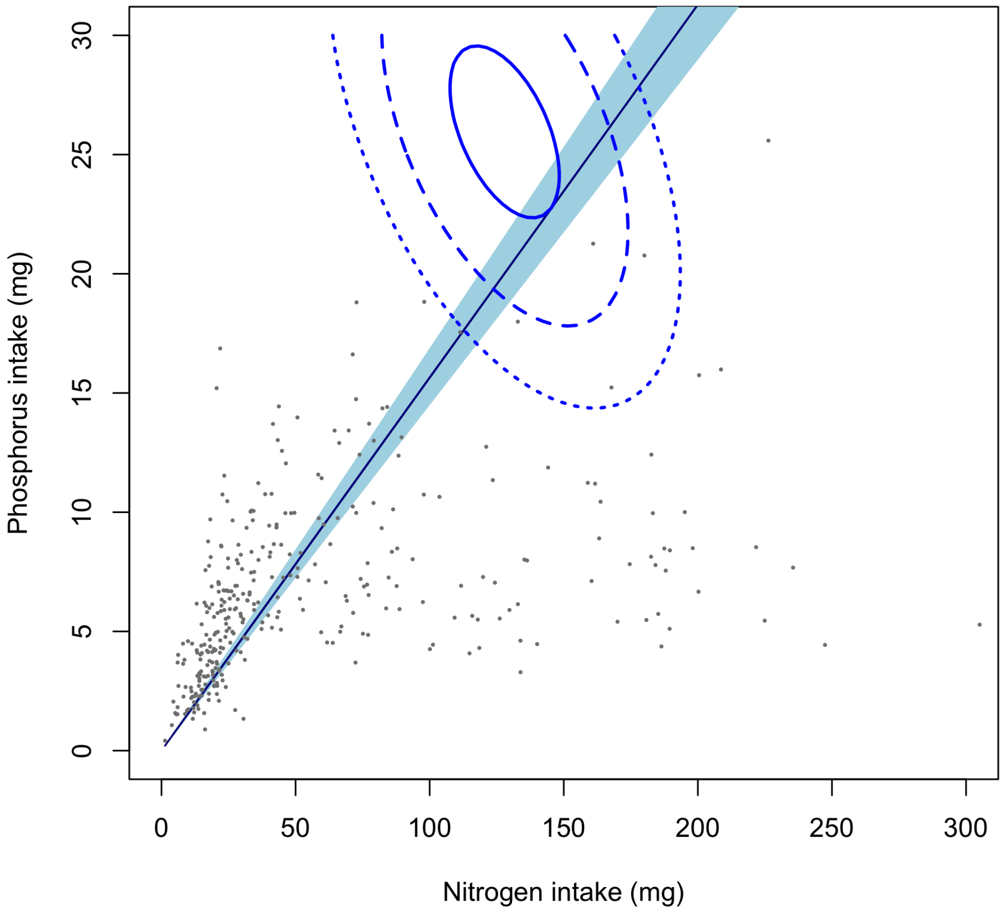


Figure 26: The top 1% of the predicted optimum from the GAM (model 4) is within the solid blue oval, the long dashed blue oval is the 5% boundary and the short dashed blue oval shows the 10% boundary. The linear black line is the orthogonal regression line from model 5, with the shaded blue area as the 95% confidence interval. The regression line goes through the 5% boundary of the predicted optimum, with the upper confidence interval going through the 1% optimum area.

Root biomass model In addition to reproductive success I measured shoot and root biomass as shown in figure 20 a and b. The root biomass is most relevant because it could be a measure for the cost currency for nutrient intake (Fisher et al., 2010). Nutrients are taken up by the roots and to understand the strategy of the plant to invest in root growth to regulate uptake depends on the species and its life history, and the environment such as the presence or absence of competitors (Hodge, 2004). Because there is no consensus about how plants regulate the uptake of nutrients, I analysed the root biomass with the same models as for fitness to see if there is a relation to nutrient uptake and how this relates to fitness. It is evident from figure 27 that root growth in relation to nutrient intake does not follow a similar pattern to the reproductive success. That would also have been a too simple assumption because the root investment is likely to be a response to nutrient and water availability. All the treatments together give an array of intake outcomes with much overlap. Because the plants with the highest fitness are not all from the same treatment, the plant shows to be able to regulate its nutrient intake to some degree.

Nutrient intake explains root growth to some extent, while root growth may explain nutrient intake. This two-way interaction should be optimal as to maximise fitness, but just root biomass is not always the right measure for uptake investment. Root hairs increase the surface area of the root and their growth is particularly responsive to phosphorus deficiency but is also shown to respond to high nitrate availability (Forde 2012). The transcription of transport proteins may also form a carbon cost. However, since the observations of Drew (1975)reflect such a clear root growth response in relation to nutrient availability, I will discuss the root biomass results of this experiment here.

Figure 27 shows that phosphorus intake and root growth have a very strong relationship. For low phosphorus availability the plant is too nutrient limited to invest in root growth, while for higher intake levels it may have the resources to invest in root proliferation while phosphorus is still limiting. For the highest phosphorus intake levels the root investment decreases which could be a sign of satiation which is also confirmed by the fitness model, that has it's optimum at the highest phosphorus intake levels.

Another notable result is the wave-like shape at high nitrogen and low (0-10mg) phosphorus intake, which coincides with a much compressed low fitness area. In this nitrogen intake area of 150-300 mg, phosphorus intake increases with root biomass, with the transition around a phosphorus intake of 5mg. At the same time, the fitness doubles within a short range of phosphorus intake. This could mean that very high nitrogen availability could trigger the transcription of phosphorus transport proteins which allows for the doubling of fitness.

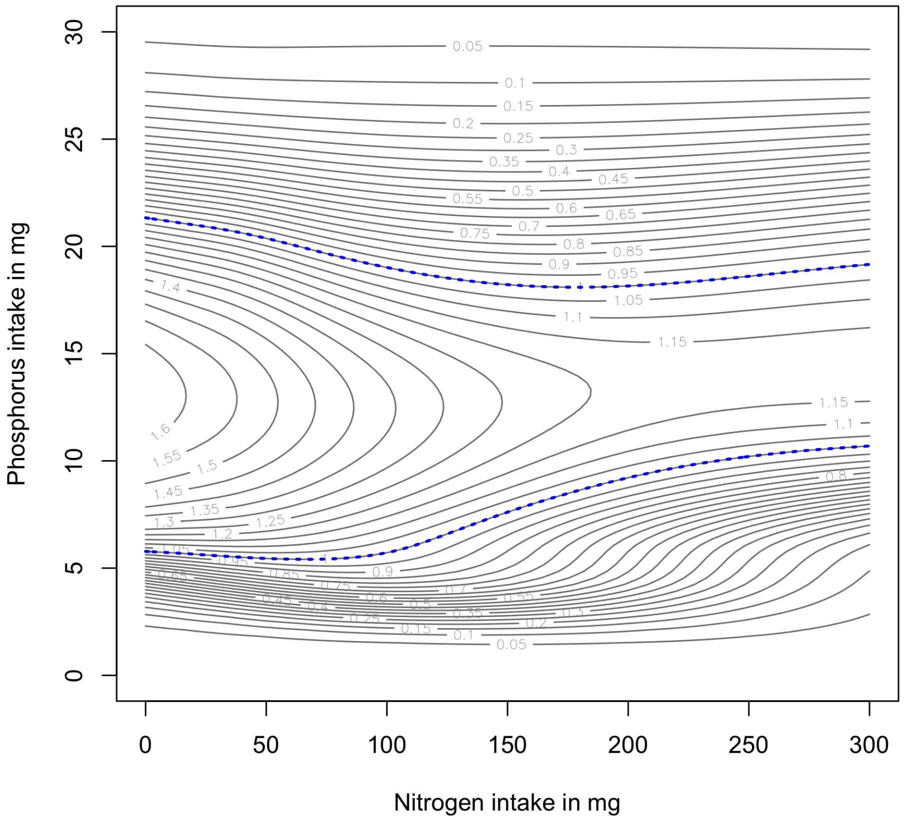


Figure 27: The predicted root biomass is shown as a function of nitrogen and phosphorus intake. The contour lines show the boundaries for root weight, with the maximum root growth near limiting nitrogen levels and medium phosphorus. The blue dashed line marks the top 1% area.

5.4 Discussion

I tested the empirical framework presented by Simpson and Raubenheimer (1993b) for the nutrition of plants. More specifically, I tested the framework for the nitrogen and phosphorus nutrition of *Poa annua* and in principle this is applicable to all plants. Most studies on uptake mechanisms test for biomass production or root growth (number of hairs or laterals) in response to nutrient limitations or excess (Fitter et al., 2002; Forde and Lorenzo, 2012; Hodge, 2004). Others study the uptake interaction between two elements under varying water availability, temperature or atmospheric CO2 concentrations (Thorup-Kristensen, 2001; Gardner, 1960; Rachmilevitch et al., 2004). Also the uptake mechanisms have been studied and the mapping of gene sets of *Arabidopsis thaliana* has provided a great many possibilities to manipulate gene transcription and help us understand plant functioning (Filleur et al., 2005; Zhang and Forde, 1998). While this is ongoing, plant functioning is complex and the interactions are numerous.

The applicability of this geometric framework surpasses the species and nutrient specific factors, and is even more defined for plants than for animals as we can build the model for the macro elements such as nitrogen and phosphorus instead of molecules. The use of this behavioural framework does not ignore the functional mechanisms, but can provide the direction for empirical studies, testing the predictions of the model. Similar to previous studies on insects, the functional interpretation provides the exact functional optima of the model species with respect to nitrogen and phosphorus. The functional consequence of suboptimal behaviour is clear, and although the range of optimal nutrient ratio increases with nutrient availability, it provides the direction to a clearly defined area of optimal intake.

In contrast to previous studies on insects, there is no defended intake target observed for *Poa annua* because the target is out of reach in relation to the nutrient supply, there is however a defended nutrient intake ratio that is represented by line *L,* only within the capabilities of the plant to downregulate nitrogen intake (Gusewell, 2004). Ammonium uptake is down regulated less effective than nitrate uptake so that the relative amount of nitrogen uptake in phosphorus deficient plants is high (Schjorring, 1986). The target intake as shown in figure 24 is the optimum predicted by the GAM model. Free-feeding animals allows them to take in exactly the preferred amount, free-feeding a plant is different and that is where the uptake mechanisms for every particular nutrient become relevant. It is unlikely that plants are adapted to the high nutrient concentrations that I provided. In the field, as long as the environment is dynamic, the plant is always limited and the level of plasticity is a trait that ensures the foraging and intake strategy is optimal in the long term. The most limiting factors would be the main drivers of speciation, while the high concentration in my treatments are not a commonly occurring situation and therefore it is possible to instigate an over-ingestion of the nutrients.

Creating a situation of over-ingestion was necessary to find the intake targets for nitrogen and phosphorus. Ammonium is imported from the soil solution via ammonium transporters in the plasma membrane of root cells, after which it is assimilated in the cytoplasm and plastid, and some amount can be stored in the vacuole. Even though the influx of ammonium is passive, the intake can be regulated by the production of transporter proteins. The intake can be actively regulated but when downregulation is insufficient toxic effects can be observed (Howitt and Udvardi, 2000). When the supplied ammonium is oxidised to nitrate by aerobic bacteria, post transcriptional regulation of uptake can occur through the activation (or lack thereof) of the ANR1 protein (Filleur et al., 2005; Walch-Liu et al., 2006). Similarly, inorganic phosphate uptake can be regulated by the production of transporter proteins, but other mechanisms are involved for post transporter protein production regulation. For phosphorus this could mean the conversion of inorganic phosphate into organic compounds, the reduction of transport through the membrane transporter proteins, and Pi loss by excretion (Schachtman et al., 1998). In both cases, excess intake of these nutrients has been observed which provides a cost and may lead to decreased fitness.

Going back to the geometric framework for foraging animals as presented by Simpson and Raubenheimer (1993b), they discuss the cost-benefit relation of over-ingesting one nutrient to ensure minimum intake of another nutrient. There is a difference between over-ingestion due to the lack of ability to downregulate intake because the intake may be passive and less controllable, and the need to over-ingest one nutrient to ensure sufficient intake of the other. This distinction is clear when animals are presented with one or more different foods that contain different defined nutrient ratios and if the target can not be reached then it will find a point of best compromise. The intake is absolute depending on the amounts of each food eaten and post-ingestive regulation can minimise the cost of the limitation or excess (Simpson and Raubenheimer, 1993b). In plants the complication is that firstly the nutrients are not necessarily taken up in the ratio they are provided. Where an animal simply eats a unit of food, the plant has different uptake mechanisms for different nutrients. Part of the uptake efficiency of one nutrient can be defined by the availability of the other (de Groot et al., 2003; Olander and Vitousek, 2000; Fujita et al., 2010) which is not simply an interaction effect by nutrient availability, but defined by the stoichiometry of the plant at its current life stage and thus its demand for each nutrient (Mankin and Fynn, 1996).

Estimating the target positions was done by providing treatment concentrations sufficiently high that it would cover the full range of limitation to excess intake although an excess of phosphorus intake was not observed. The target position could still be defined based on the data, even though the absolute target intake was not defended. The results from this experiment on *Poa annua* shows that a fitness cost occurs for both high levels of nitrogen and phosphorus.

The tilted ellipse in figure 24 shows that an excess uptake of either nutrient exclusively substitutes to some extent for a deficiency of the other, but excess of both nutrients annuls this effect. Note that the ellipse is the model prediction, but there were very few observed data points that match these situations. This shows the ability of a plant to downregulate intake in most cases and that even when nutrient availability was high, nutrient intake did not consistently meet the target. This relates to the law of the minimum that success is determined by the most limiting nutrient (Hooker, 1917), and the demand to increase its fitness is now limited by an abiotic factor that is not under study. That is not unlikely since the background nutrition was not optimised for every nutrient and, like what I found for nitrogen in chapter 4, there may well be a much higher demand for one of those nutrients than estimated as the 50% in Hewitt (1966).

For suboptimal nitrogen and phosphorus intake, I found the regression line L which also goes through the optimum. There are fewer observations deviating from this line with higher P/N ratio than with a lower P/N ratio. Therefore it seems *Poa annua* is less capable of downregulating ammonium intake than phosphorus intake. This is not surprising since the mechanisms to downregulate intake after transporter protein transcription, are limited and the application of high nitrogen concentrations was ongoing throughout the experiment (Gusewell, 2004). On the other hand, the excretion of inorganic phosphorus, which also avoids the cost of assimilation, can go up to 70% of the influx (Bieleski, 1973). However, when we look at the treatment ratio's (beige diagonal lines in figure 24), the preferred P/N intake ratio is much higher than that of most treatments. Hence, only the plants with the highest P/N ratio treatment were given near optimal nutrient ratios. For all other treatments the plants had to downregulate nitrogen intake of which its ability showed to be limited.

Another explanation could lie in the mechanism of enzyme production in relation to nutrient availability. Chitinase and phosphatase have the function of mineralising nitrogen and phosphorus (respectively) and are therefore a means to making the nutrients available for uptake (Gusewell, 2004). Olander and Vitousek (2000); Fujita et al. (2010) showed that increased nitrogen availability decreased chitinase activity in the rhizosphere, and increased phosphatase activity. Vice versa, phosphorus availability did decrease phosphatase activity but had no effect on chitinase activity. In my experiment the activity of these enzymes would not have had much effect because the plants were growing on a sand-vermiculite substrate without any organically bound nutrients. That does not mean however that the plant could not produce these enzymes in an attempt to increase uptake of the limiting nutrient. The plants in my experiment with high nitrogen concentration could thus have lost phosphorus by both inorganic phosphorus excretion and by phosphatase excretion stimulated by nitrogen availability, while nitrogen concentration was not affected by phosphorous availability.

The mechanisms behind the observations can all be explained, but what is more important is that there appears to be a clearly defined intake target for the model plant, and a preferred intake ratio of nitrogen and phosphorus. Even though there are many interactions and cofactors responsible for immediate unaccounted responses and thus variation, the theoretical background predicting a linear feeding line towards the intake target is valid. Substitutability of nutrients could complicate the model, and should therefore be further investigated. My question now is whether the theory still holds when the plant is in an extreme state that is far off this line of preferred nutrient ratio. This would be incurred by applying treatments that exceed the ability of the plant to downregulate intake, after which they are provided with the limiting nutrient. The theory predicts that the plant should only take in the limiting nutrient as to reach the preferred nutrient ratio, rather that taking up the sufficiently available nutrient as well as to go directly to the intake target. One limitation of this theory for plant uptake, is that nutrients are taken up simultaneously and downregulation may incur as much of a cost as continuing the uptake of low levels.

From the root biomass data it was also confirmed that regulation is not only done by root growth. Phosphorus, rather than nitrogen, influences the production of root biomass. Although uptake and transport mechanisms in the plant are complex for both nitrogen and phosphorus, this response is easily understood as also life history predicts that the plant needs to forage for phosphorus as long as it is limiting Williamson et al. (2001). This idea however contradicts Drew (1975) and Robinson (1996), where, even though higher proliferation responses were expected for immobile nutrients, also proliferation responses were found for the mobile nutrients. However, an increase in root length density could be more important for the uptake of immobile nutrients than for mobile nutrients (Fitter et al., 2002), so the root proliferation response to mobile nutrients could well be explained by the increased demand for other (that is immobile) nutrients when availability of the mobile nutrient increases (Mankin and Fynn, 1996), although Fujita et al. (2010) showed a reduced specific root length and root mass ratio with increased nitrogen availability and demonstrated that phosphatase activity is increased to stimulate phosphorus uptake and maintain a stable N-P ratio. Contradictive results between studies can be explained by the presence and absence of competition, where root proliferation is important for nitrate uptake when growing in competition, while it is not when growing as individual Hodge (2004). Also, Grime and Mackey (2002) found a low root foraging precision in the monocotyledons, which they reason is likely a result of the dependence of monocotyledons on adventitious roots and augment their root systems by producing a network of fine roots near the soil surface. This inefficiency could be subject to a recovery trade-off, since habitats with turf-grasses are often grazed and trampled by large herbivore grazers Grime and Mackey (2002).

Part III

Testing the two-food model

6 Split root experiment - biomass

6.1 Introduction

Regulating nutrient intake in heterogeneous soils is a matter of integrating the information from the various local modules at the system level, and responding both locally and systemically. Even without a brain a plant is capable of doing so and this regulative ability could be explained as dietary self selection. One of the earliest studies on dietary self selection was done by Evvard (1915) on the free-choice system of feeding swine. He wondered whether the appetite of swine was a reliable indication for their bodily needs, and if they could efficiently balance their own ration. During the century that followed, many researchers have asked the same question for rats (Abrams et al., 1949; Osborne et al., 1918; Richter et al., 1938), Grasshoppers (Simpson and Abisgold, 1985; Raubenheimer and Simpson, 1997, 2003; Simpson and Raubenheimer, 1993a, 2001; Raubenheimer and Simpson, 1993; Chambers et al., 1995) and more. In these examples, the organisms have a centre, the brain, from which nutrient state, demand and intake are integrated. Dussutour et al. (2010) found that also the slime mould which does not have a brain, can make complex nutritional decisions, growing into nutrient patches with variable quality in such proportion that it meets its precise nutrient requirements. As a result of the free choice system the Iowa pigs of Evvard (1915) showed more weight gain in a shorter time than their hand-fed Illinois counterparts. Therefore it can be said that they have the capacity to maintain a nutrient balance by selective foraging. In chapter 5 I analysed the free-feeding system of plants, that is all the treatments with sufficient to excess nutrient availability, and I found they were balancing nutrient intake in the ratio of the predicted optimum.

In the literature, the theories on selective feeding have refined to consider the composition of foods more specifically, addressing the possibility of a forager to over ingest one nutrient in order to reach a minimum intake of another, to minimise the fitness cost related to either

(Raubenheimer et al., 2009). Based on the relation between minimising fitness cost, hence maximising fitness, and nutrient intake, a feeding strategy was mathematically derived by Houston et al. (2011) (equations 10, 11, 12 and 13, Chapter 1). The intake target in the nutrient space is a given as the optimum in the fitness landscape and the optimal strategy to reach this target is to feed on the food that consists of the nutrients under study in the ratio as the intake target. This provides an optimal feeding rail, line L. However, often the available foods do not have this optimal composition and the organism needs to feed on the food that lets them reach the optimal nutrient ratio state, that is, reach a state on line L. So when the nutrient state of an organism is somewhere below or above this line in the nutrient space, it should be exclusively feeding on the food that allows the state to change towards the optimal feeding line (mathematical background in Chapter 1). In figure 7, Houston et al. (2011) represented the various possible feeding strategies, based on the food compositions available. Once the line is reached the organisms should switch between the foods to stay near the line.

In Chapter 5, I calculated the ratio of the optimal feeding line, based on the defended nutrient intake ratio which leads to the optimal intake target. Based on this line I can test if the selective feeding strategy of *Poa annua* is as predicted by the theory. Figure 28 shows the basic theory for when two foods are available containing only one nutrient. Translating this to the foraging of a plant these two foods are defined as two separate patches containing only one nutrient or the other. Applying the theory to plants is complicated, because once the roots have proliferated into both patches it can take up the nutrients simultaneously. Also, as we have seen in Chapter 5 there is an interaction in the uptake of phosphorus and nitrogen.

The situation where the plant has two patches available with a combination of nutrients in given in figure 29. To ensure optimal nutrient intake, that is feeding along line L, the prediction is that the plant should balance its root growth in the patches in a way that it can take the nutrients up in the preferred ratio. Once the roots have grown and it reaches the optimal feeding rail, the switching becomes an invisible process which could be regulated by the other uptake mechanisms such as the regulation of transporter proteins.

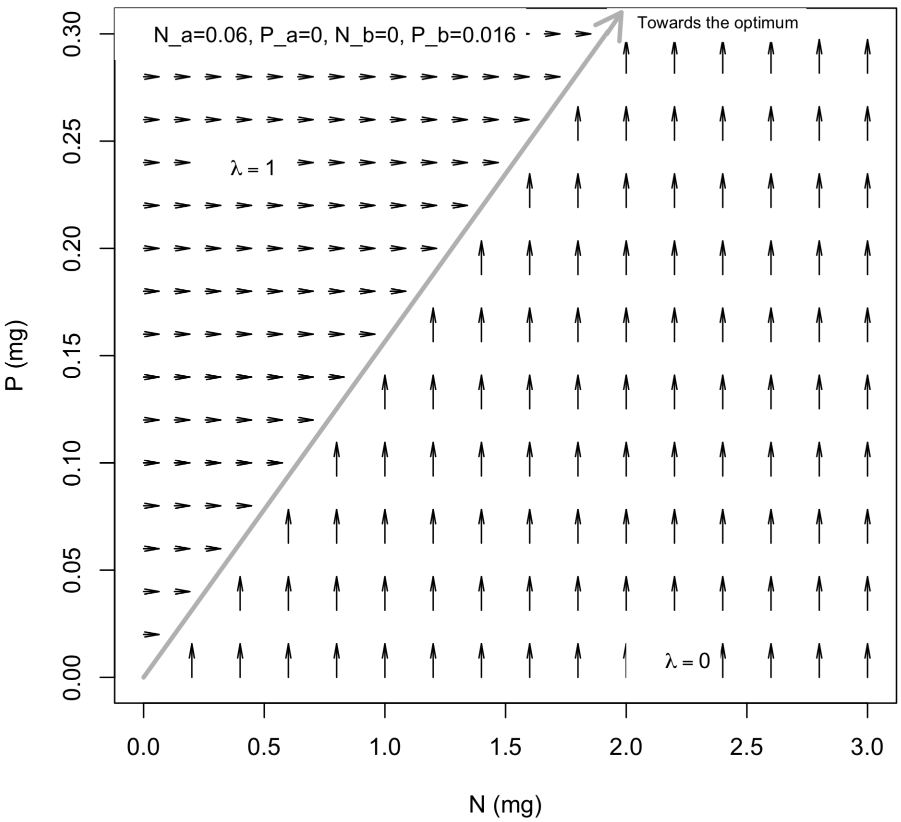


Figure 28: Optimal feeding rail for *Poa annua.* The arrows reflect the direction of feeding that is preferred when both nutrients are available and the plant can take them up independently. In the $\lambda = 1$ area, the plant should be taking up nitrogen only, and in the $\lambda = 0$ area the plant should be taking up phosphorus. N_a: The amount of nitrogen in patch a. P_a: The amount of phosphorus in patch a. N_b: The amount of nitrogen in patch b. P_b: The amount of phosphorus in patch b. Adapted from Houston et al. (2011), figure 3.

From this theory I derived two questions based on the findings in Chapter 5:

1. Is exclusive feeding observed in any suboptimal nutrient state that is not in the ratio of the optimal feeding rail in the model species *Poa annua?*
2. Is the plant able to regulate nitrogen intake to defend the optimal intake ratio of line *L,* when provided with different ratios of excess nitrogen and phosphorus availability?

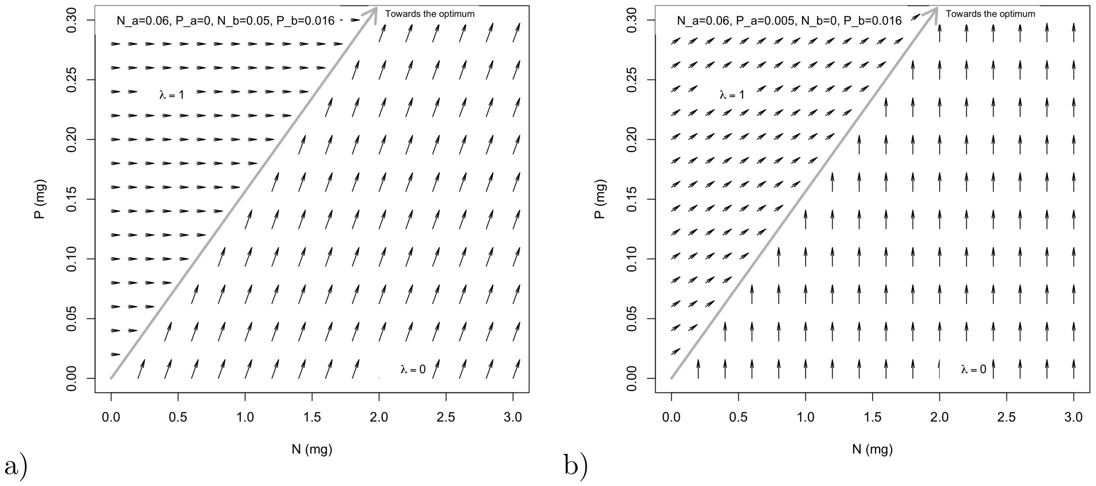


Figure 29: Optimal feeding strategy for the situation of patch a containing nitrogen alone, and patch b. containing nitrogen and phosphorus. (a). b) Reflects the situation in which patch a contains both nitrogen and phosphorus, and patch b contains a combination of nitrogen and phosphorus. N_a: The amount of nitrogen in patch a. P_a: The amount of phosphorus in patch a. N_b: The amount of nitrogen in patch b. P_b: The amount of phosphorus in patch b. Adjusted from Houston et al. (2011), figure 3.

To answer these questions I set up two sets of conceptually different experiments. The first set was based on forcing the plants into a nutrient state with a higher and lower N/P ratio than line L and subsequently providing either a control treatment with no nutrients, the sufficiently available nutrient, or the limiting nutrient. The second set of experiments are based on the concept of free-feeding the plant in different N/P ratio's with excess nutrient availability, with the nutrients exclusively supplied in the separate patches. The root proliferation balance in the patches should be relative to the demand for each nutrient, the demand is based on the slope of line L and is verified with final nutrient uptake measurements.

1) Is exclusive feeding observed in any suboptimal nutrient state that is not in the ratio of the optimal feeding rail? To test the hypothesis that plants will feed on the most deficient nutrient exclusively, I set up two experiments, each with three treatments. These two experiments are essentially the same, but with the nutrients phosphorus and nitrogen reversed. I grew the plants with a nitrogen or phosphorus exclusively, both with a background solution for the other essential elements. This was to create plants with a sufficient nitrogen and deficient phosphorus state (State 1), and plants with a sufficient phosphorus and deficient nitrogen state (State 2), and these are now referred to as State 1 experiment, and State 2 experiment (table 15). After the plants were brought into their required state, I analysed some of them to determine their nutrient state. The other plants were then grouped into three categories: 1) the treatment group to receive phosphorus, 2) the treatment group to receive nitrogen, and 3) the control group that only received the background nutrients (15). The plants were moved into a split root pot with a side A for the background solution, and side B for the treatment solution (table 15).

Table 15: Experiment organisation

	Pre- treatment		Space	Group	Treatment	
	N	P			N	P
State 1	High	Low	$\lambda = 0$	1.1	Low	High
				1.2	High	Low
				1.3	Low	Low
State 2	Low	High	$\lambda = 1$	2.1	Low	High
				2.2	High	Low
				2.3	Low	Low

The predictions of these State 1 and State 2 experiments are visualised in figure 30a. What I analysed is the actual state of State 1 and 2 after three weeks of pre-treatment, and the state change after three weeks of treatment. To see if the foraging effort for the uptake of the nutrients is reflected in root biomass, the nutrient treatments were given in side B of the split root pot. What is more important than the increase in nutrient state of the initially deficient nutrient, is that there should be no increase in nutrient state of the sufficient nutrient, or at least not bigger than in the control treatment, and it should not have grown more roots in side B than in side A. For an overview of the expectations of root investment and nutrient intake see table 16.

Table 16: Predictions State 1 and 2

Group	Resulting nutrient state		Root investment side B
	Nitrogen	Phosphorus	
1.1	No change	Increase	Larger than in side A
1.2	No change	No change	Same as side A
1.3	No change	No change	Same as side A
2.1	No change	No change	Same as side A
2.2	Increase	No change	Larger than in side A
2.3	No change	No change	Same as side A

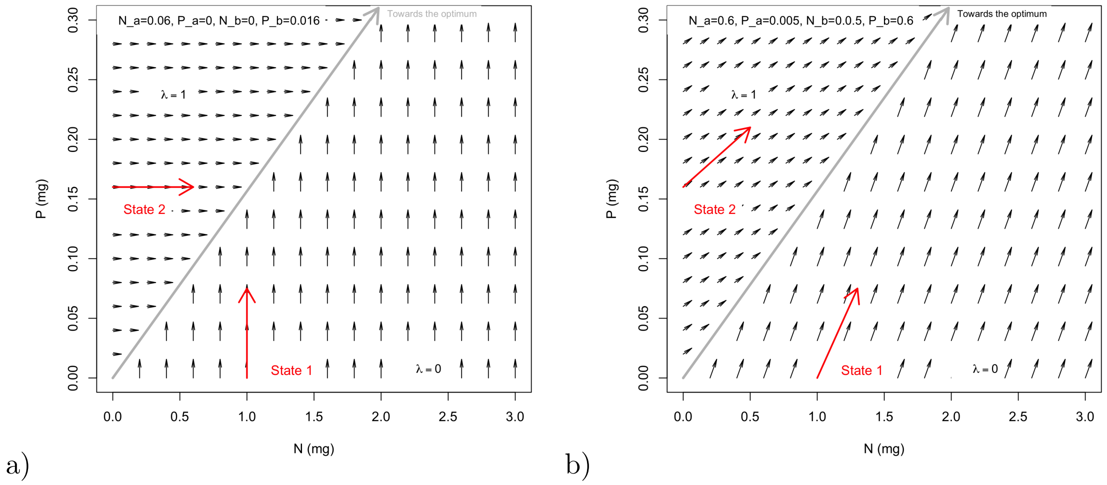


Figure 30: Expectations for the state-change of plants in State 1, when supplied with phosphorus, and State 2 when supplied with nitrogen. a) Situation where the state change is in one nutrient exclusively. b) State change strategy when the nutrient rich patches contain a combination of nutrients and these are take up in interaction. N_a: The amount of nitrogen in patch a. P_a: The amount of phosphorus in patch a. N_b: The amount of nitrogen in patch b. P_b: The amount of phosphorus in patch b. Adjusted from Houston et al. (2011), figure 3.

A complication to these predictions is that I used the substrate from the pre-treatment in the split root treatments. That means that it is likely that some traces of the pre-treatment nutrients are still available. This results in a combination of nutrients in side B when supplied with the deficient nutrient in the split root treatment, which could lead to the situation in figure 30b, adjusted from 7c, if nutrients are take up in interaction. Even if the nutrient rich patch (side B) has both nutrients available, I expect the state change to follow the prediction as in figure30a and table 16, because the plant can downregulate intake of the sufficient nutrient, and it should do so because even though the roots can double for the uptake of both nutrients, nutrient assimilation and transport is costly.

It needs to be noted that the plants will not, in any situation, start feeding along the optimal feeding rail, simply because they are only provided one nutrient exclusively. If they would also take up the trace nutrients from the pre-treatment, that will be reflected in the state change while moving towards line *L* as in figure 30b.

2) Is the plant able to regulate nitrogen intake to defend the optimal intake ratio of line *L*? For this set of experiments I used plants that were in a nutrient state near the origin, on the optimal feeding rail. I split the roots to grow into two patches, in which phosphorus and nitrogen were supplied exclusively. The phosphorus concentration was equal to the highest concentration used in Chapter 5, 0.213 mM and I used the three highest nitrogen concentrations for the different treatments, that is: 6.418, 12.835 and 25.67 mM. If plants regulate their nitrogen uptake through root growth, it should grow roots in increasing proportions in the phosphorus patch, to up regulate phosphorus intake and balance the intake ratio according to nitrogen availability. Additionally, it could downregulate root growth into the nitrogen patch to regulate nitrogen uptake according to the phosphorus availability, given the nitrogen concentration.

I grew the plants for three weeks with a background solution without nitrogen and phosphorus. This was necessary to follow a comparable procedure as for question 1. Some plants were analysed at this point to confirm their nutrient state, the remaining plants were grouped into three treatments with equal phosphorus availability, and three nitrogen concentrations. The expectation was that the final nutrient state would be in the optimal feeding ratio, and root investment in the phosphorus rich patch would be up regulated according to nitrogen availability.

6.2 Methods

Growth conditions From the previous experiments I learned that variability with this model species can be high, so I chose to have 20 replicates for every pre-treatment, and treatment. For the first set of experiments this resulted in 20 samples per state (state 1 and 2) and 20 samples per treatment (nitrogen, phosphorus and control), hence 160 plants. For the second three experiments I only used 20 plants for the state, and 20 plants for each treatment, resulting in 80 plants.

I sowed the seeds on a 50/50 sand-vermiculite mixed substrate. After the seeds had germinated, I planted 240 seedlings in 12ø pots on the same sand-vermiculite mixed substrate and I applied the pre-treatment for three weeks. After three weeks I took 20 replicates from each state for analysis, the remaining plants were transplanted into the split root pots. See Appendix B.1 for photos of the split root setup.

Split root pots I created the split root pots by taping a lunch bag around a beaker and piercing the bottom with 8 holes. The size of every pot part was half the volume of the 12ø pots the seedlings received their treatment in. Two pot parts were taped together to form one plant pot with two compartments. I used the substrate from the pre-treatment to fill the compartments of the split root setup, and placed the seedling on the edge between the compartments. Seedlings had 3-5 small roots at this stage, which I placed approximately evenly on both sides. Treatment application was done for three weeks, twice weekly with 25 ml per treatment side. Throughout the experiment, humidity and CO_2 levels were ambient, light was controlled for a 12 hour day length and temperature was 22 degrees C. in the day and 20 degrees C. at night.

Treatments question 1 The treatment concentrations are found in table 17, for the background concentrations I refer to Chapter 5, table1 and 2.

Table 17: Experiment organisation

	Pre- treatment		Space	Group	Treatment side A	Treatment side B	
	N	P				N	P
	Concentration (mM)					Concentration (mM)	
				1.1		0	0.213
State 1	25.67	0	$\lambda = 0$	1.2	Background	25.67	0
				1.3		0	0
				2.1		0	0.213
State 2	0	0.213	$\lambda = 1$	2.2	Background	25.67	0
				2.3		0	0

Treatments question 2 The treatments for this set of experiments are given in table 18. During both the pre-treatment and the treatment period I applied the background solutions as in table 1 and 2.

Table 18: Experiment organisation

	Pre- treatment		Space	Group	Treatment side A	Treatment side B
	N	P			P	N
	Concentration (mM)				Concentration (mM)	
				3.1	0.213	6.418
State	0	0	Origin	3.2	0.213	12.835
				3.3	0.213	25.67

Measurements After three weeks of pre-treatment and three weeks of treatment the experiment was terminated. I separated the roots and shoots, washed the roots and dried all the material at 70º for several days. There were no plants that had started flowering so seed handling and weight is not relevant in this experiment. For nitrogen and phosphorus content measurement I refer to the Chapter 5 methods. Because the plants were tiny, only the weight of the separate root compartments was registered, it was not possible to analyse nutrient content of the separate root parts and shoots so the whole plant was processed in one sample for nitrogen and phosphorus analysis.

Statistical analyses I did all the analyses and plot building in R 3.2.2 GUI 1.66. The biomass data were not normally distributed, so group-wise comparisons were always done with the Wilcoxon rank sum test with the wilcox.test() function.

6.3 Results

1) Is exclusive feeding observed in any nutrient state where $\triangle(N,P) \neq \mathbf{0}$ The plants that were brought into a phosphorus deficient state (referred to as state 1) were provided three different treatments (figure 31). The plants that received nitrogen did not differ in their final nitrogen state from the control plants and the phosphorus state of the control plants was slightly higher. Phosphorus intake was higher in the plants that received phosphorus treatment than those that did not (19). The key is that nitrogen intake of the plants that received the phosphorus treatment did not differ from the control and nitrogen treatments. The mean state of the phosphorus treatment is not exactly on the line, however the variance is high and line L goes through the 1sd from the mean of the phosphorus treatment plants.

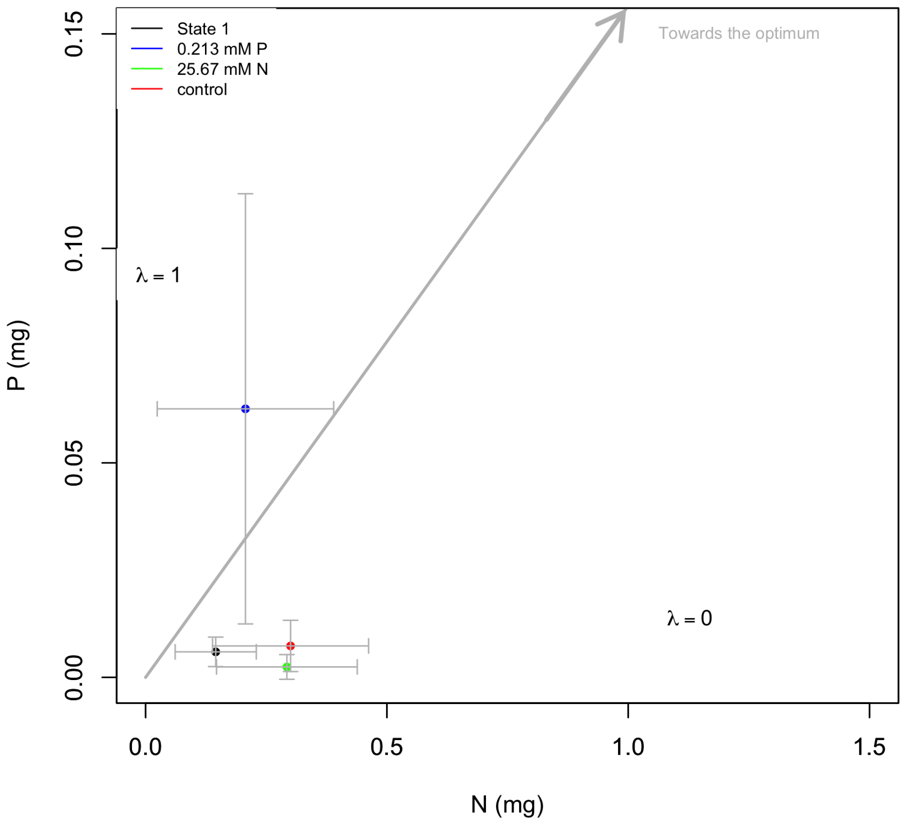


Figure 31: The coloured dots reflect the mean nutrient composition (+/- 1sd) placing the state of every situation in the nutrient space. The diagonal line is the optimal intake ratio (line *L*) as derived in chapter 5. The black point refers to the nutrient state of the plants that received a nitrogen with background solution, and lacking phosphorus, for three weeks. This is now the start-state which was forced to be in the $\lambda = 0$ space. The green, red and blue point all refer to plants that received a treatment for a subsequent three weeks after having received the state 1 solution for the first 3 weeks. The green point shows the mean of plants that received a nitrogen solution solution, the red point is the mean for the plants that received a background solution only. The blue point shows the mean intake values of the plants that received the phosphorus treatment. The prediction was that the control (red) and the nitrogen treatment (green) would not differ, and that the location of the blue point would be on the optimal feeding rail.

Table 19: State 1 statistics

Treatment	Figure 31	Mean nitrogen intake (mg)	Difference p-value	Mean phosphorus intake (mg)	Difference p-value
State 1		0.145 ± 0.084		0.006 ± 0.003	
Nitrogen treatment	Green	0.293 ± 0.146	0.467	0.002 ± 0.003	< 0.01
Control	Red	0.300 ± 0.162	0.111	0.007 ± 0.006	< 0.001
Phosphorus treatment	Blue	0.207 ± 0.183		0.063 ± 0.050	

Data were not normally distributed.

Test for significant differences was done with a Shapiro Wilcoxon rank-sum test in R.

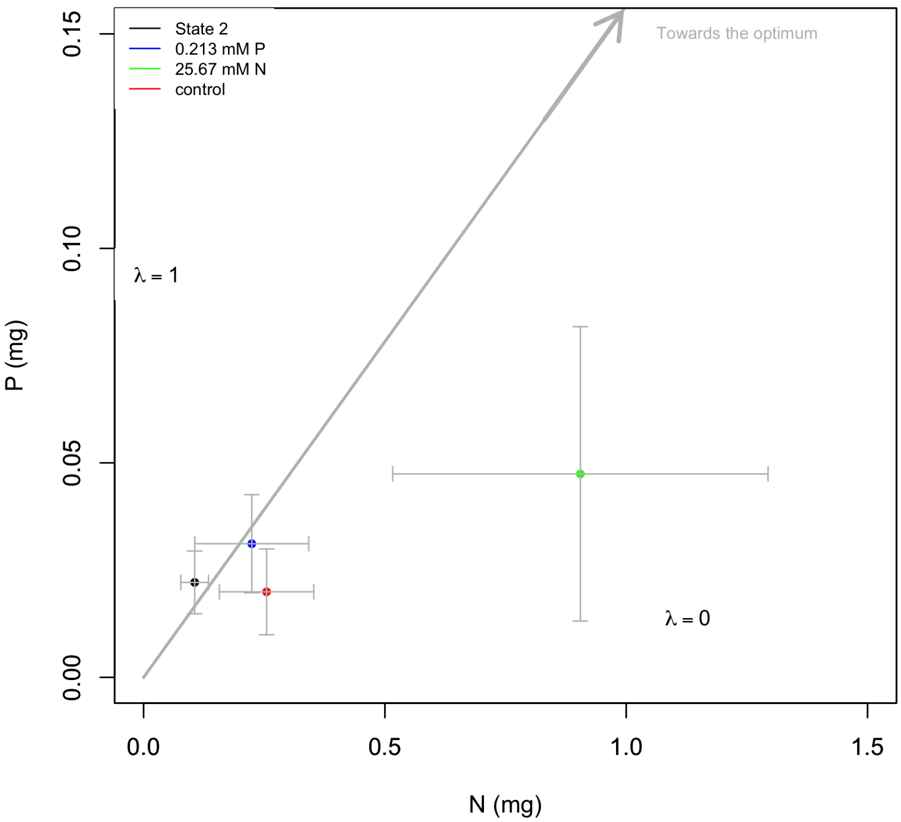


Figure 32: The coloured dots reflect the mean nutrient composition (+/- 1sd) placing the state of every situation in the nutrient space. The diagonal line is the optimal intake ratio (line *L*) as derived in chapter 5. The black dot refers to the nutrient state of the plants that received a phosphorus with background solution without any nitrogen, for three weeks. This is now the start-state which I intended to force into the $\lambda = 1$ space. The green, red and blue dot all refer to plants that received a treatment for an additional three weeks after having received the state 2 solution for the first 3 weeks. The green point is the mean intake of plants that received a nitrogen solution, the red dot is the mean for the plants that received a background solution only. The blue point shows the mean intake values of the plants that received the phosphorus treatment. The prediction was that the control (red) and phosphorus (blue) treatment would not differ, and that the location of the green point would be on the optimal feeding rail.

Table 20: State 2 statistics

Treatment	Figure 32	Mean nitrogen intake (mg)	Difference p-value		Mean phosphorus intake (mg)	Difference p-value	
State 2		0.106 ± 0.029			0.022 ± 0.007		
Nitrogen treatment	Green	0.905 ± 0.389	< 0.001		0.047 ± 0.034	< 0.001	
Control	Red	0.255 ± 0.098		0.483	0.020 ± 0.010		0.002
Phosphorus treatment	Blue	0.224 ± 0.118			0.031 ± 0.011		

Data were not normally distributed.

Test for significant differences was done with a Shapiro Wilcoxon rank-sum test in R.

The opposite of bringing plants in a phosphorus deficient state (figure 31) was to bring the plants in the nitrogen deficient state which is shown in figure 32. After those three weeks of bringing the plant in state 2, the nutrient state is well near the optimal feeding rail. Adding more phosphorus made the state of these plants move up along the feeding rail, while the control treatment was showing a lower phosphorus intake level. The nitrogen treatment however had a both higher nitrogen and phosphorus intake than the control group20, but the phosphorus intake was not higher than that of the phosphorus treatment (not shown).

In both situations the nutrient state at three weeks represented the pre-treatment they had received, but state 1 was more phosphorus limited than state 2 was for nitrogen. Interestingly, the situations where nitrogen was expected to be limiting (state 2 and state 2+phosphorus) the plant's nutrient states were on the optimal feeding rail. Adding nitrogen took them far into the $\lambda = 0$ space which was thought to be suboptimal based on the predicted optimum from chapter 5. To the question if the plant is feeding exclusively in the patch containing the nutrient it is limited of, there are two answers depending on the nutrient relation. Phosphorus deficient plants took up more phosphorus when supplied with phosphorus than phosphorus sufficient plants, and did not increase nitrogen uptake. So yes, in this situation the plant was feeding selectively on the deficient nutrient. However, nitrogen deficient plants took up both nitrogen and phosphorous when supplied with nitrogen. The phosphorus intake is likely a result of trace nutrients available from the pre-treatment. So nitrogen uptake does not happen exclusively and also seems to be less controlled since it deviates from the optimal feeding rail more than the control treatment does.

2) Is the plant able to regulate nitrogen and phosphorus intake to defend the optimal intake ratio of line *L*? In this experiment I created three treatments in which the plants received high concentrations of both nitrogen and phosphorus in three different ratio's. This situation should reflect the free-feeding situation as seen in animal nutrition. The three different ratio's supplied mimic the different food compositions as in animal studies, the difference being that the plant can extract different amounts of each nutrient provided.

The N-P ratio of the treatment solution increased, and with that the nitrogen uptake increased (33), but the phosphorus intake was not increased, so N-P ratio of the nutrient state increased.

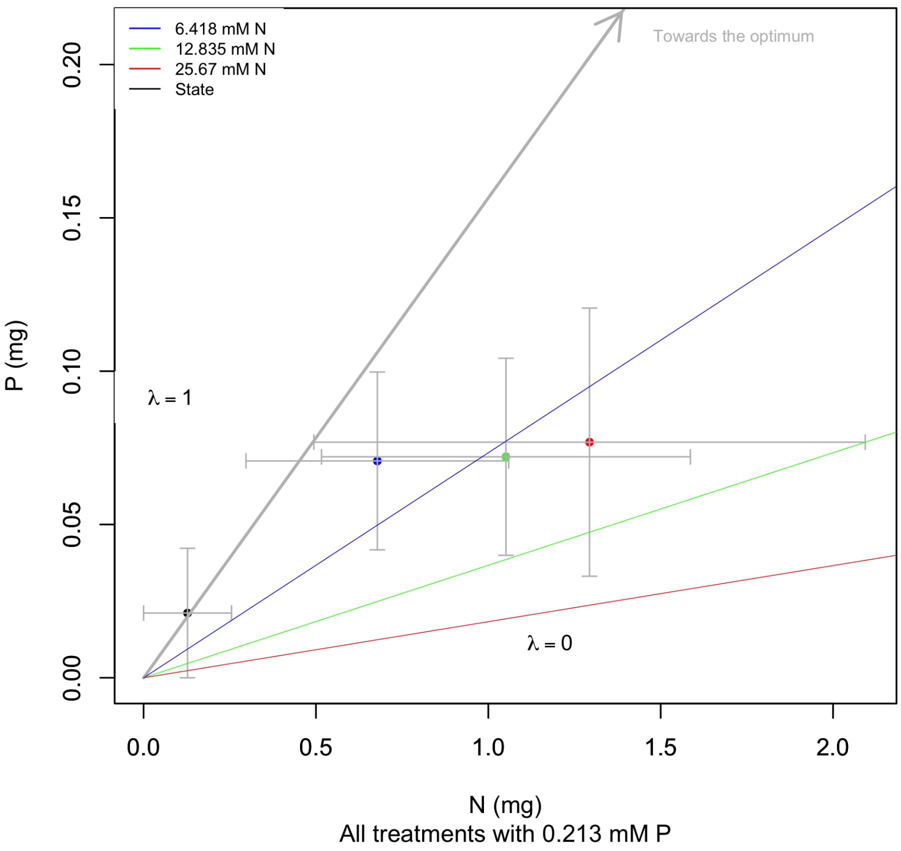


Figure 33: The coloured dots reflect the mean nutrient composition (+/- 1sd) placing the state of every treatment in the nutrient space. The grey diagonal line is the optimal intake ratio (line *L*) as derived in chapter 5. The black dot refers to the nutrient state of the plants that received no nutrients for the first three weeks, only a background solution. The state of these thee week old plants is on the optimal feeding rail which is as expected. The green, red and blue points refer to the different nutrient ratio's that were applied after the pre-treatment. The blue, green and red points are the resulting states of the different treatments with increasing N-P ratio, with 6.42, 12.84 and 25,67 mM nitrogen respectively. The nutrient rails representing the nutrient ratio of the treatments are shown as the diagonal lines in matching colours. The prediction was that nutrient state of every treatment was on the optimal feeding rail, with higher phosphorus intake for higher nitrogen availability.

Table 21: State 3 statistics

Treatment	Figure 33	Mean nitrogen intake (mg)	Difference p-value		Mean phosphorus intake (mg)	Difference p-value	
State 3		0.126 ± 0.057			0.021 ± 0.010		
N 6.418 mM	Blue	0.678 ± 0.381		↖	0.071 ± 0.029		↖
			0.044			0.857	
N 12.835 mM	Green	1.051 ± 0.535		0.013	0.072 ± 0.032		0.727
			0.398			0.655	
N 25.67 mM	Red	1.294 ± 0.800		↙	0.077 ± 0.044		↙

Data were not normally distributed.

Test for significant differences was done with a Shapiro Wilcoxon rank-sum test in R.

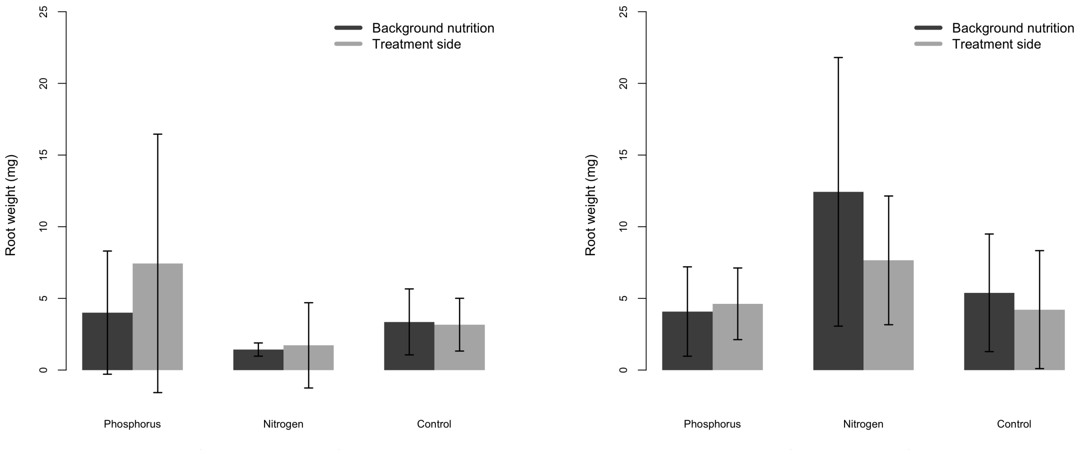
The doubling of the nitrogen availability did not cause a significant increase in nitrogen uptake, but a four-fold did (table 21). Like with the previous two experiments, the variation in nitrogen intake is large and there is much overlap of the standard deviation of different treatments. Interestingly, the phosphorus uptake did not at increase even though the phosphorus concentration was 0.213 mM. From chapter 5 I expected that the optimal nutrient intake is around 130 mg nitrogen and 25 mg phosphorus. In figure 24 we also saw many data points with a higher N-P ratio than expected from the optimum, and I related this to insufficient phosphorus availability.

Now, phosphorus availability is not an issue unless there are constraints to the phosphorus uptake to meet the demand. In this case the plant should downregulate nitrogen intake until phosphorus intake is up regulated, and it did not do so. It is unclear if the plant is not able to downregulate intake, or if it has the strategy to take up ammonia in excess and store it until phosphorus intake meets nitrogen state. However, this strategy did show to compromise fitness.

Root growth Part of the optimal intake strategy is also the cost expenditure or carbon allocation strategy. Even though there are several different carbon costs to nutrient uptake, such as the expense on assimilation and transporter protein activity, the expense of root biomass allocation would be a large cost and measurable in terms of directed investment towards the uptake of a specific nutrient.

The split root pots allowed me to measure root growth in response to each nutrient exclusively and the results are given in figure 34. If foraging for a nutrient is reflected in root proliferation, and nutrient uptake is exclusive for one nutrient until the optimal feeding rail is reached, the plants should grow they roots in the nutrient pool that is in highest demand exclusively.

Exclusive root investment is not observed in any of the split root setups. The total root growth was increased when plants were provided with the limiting nutrient compared to the control groups: State 1, the total root biomass of the nitrogen treatment was higher than that of the plants in the control treatment ($p < 0.01$). State 2, the total root biomass of the phosphorus treatment was higher than that of the plants in the control treatment ($p < 0.001$).



a) b)

Figure 34: The root investment (± 1 sd) into the background solution (dark grey) and the treatment patch (light grey). The plants were pre-treated with a zero phosphorus solution (a) creating state 1 or a zero nitrogen solution (b). Subsequently they were provided with phosphorus, nitrogen or a control treatment lacking either. The expectation was that the plants would proliferate more roots in the patch providing the nutrient they were deficient of. For both phosphorus and nitrogen root growth seems to increase, roots may grow more in the phosphorus patch when phosphorus is needed but for nitrogen root growth in the background solution seemed larger. The variation is so large that no significant differences can be found.

Table 22: State 1 and 2 statistics for root growth

Treatment		Mean root biomass (g) background (side A)	Difference p-value	Mean root biomass (g) treatment (side B)
State1	Figure 34 a.			
Phosphorus		4.007 ± 4.297	← 0.395 →	7.443 ± 9.014
Nitrogen		1.431 ± 0.461	0.051	1.723 ± 2.974
Control		3.360 ± 2.300	0.756	3.167 ± 2.300
State 2	Figure 34 b.			
Phosphorus		4.083 ± 3.117	0.602	4.622 ± 2.499
Nitrogen		12.433 ± 9.369	0.147	7.653 ± 4.490
Control		5.389 ± 4.102	0.069	4.217 ± 4.114

Data were not normally distributed.

Test for significant differences was done with a Shapiro Wilcoxon rank-sum test in R.

In figure 35 we see that there are no differences in root proliferation between the nitrogen and phosphorus patches. Also between the treatments there are no differences between total root biomass. However, total plant biomass increased between the treatments with 6.42 and 12.84 mM nitrogen (from 34.4 to 48.2 g, p = 0.014), but I found no difference with the highest nitrogen treatment. A recurring finding throughout chapter 5 and 6 is the increase in variation with increasing nitrogen concentration in the soil.

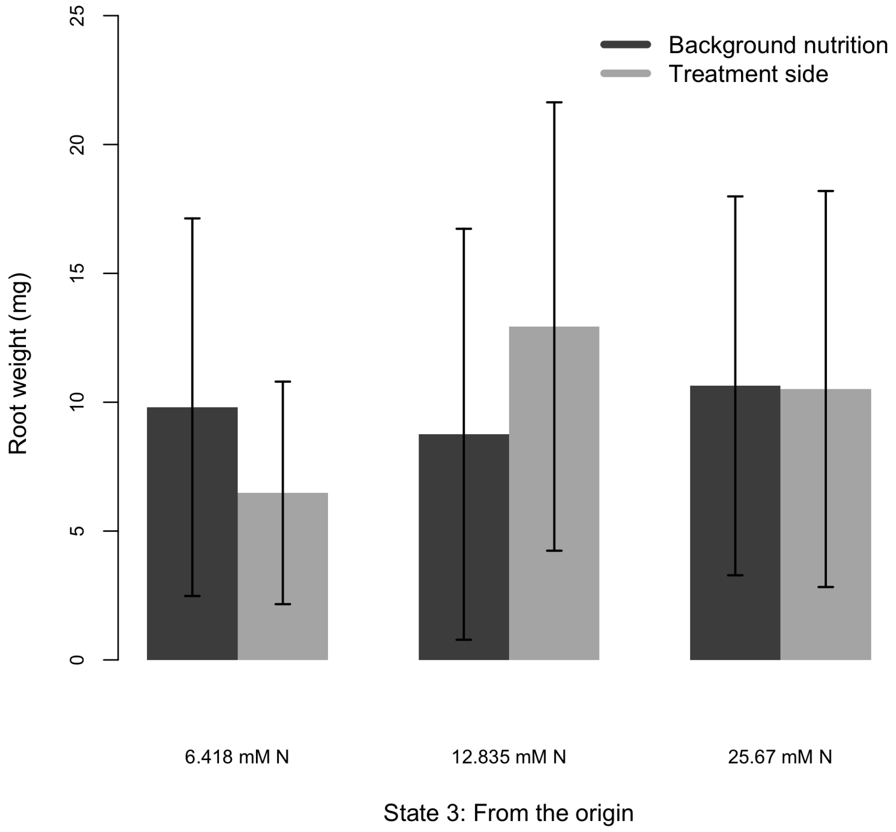


Figure 35: The root investment (± 1 sd) into the phosphorus solution (dark grey) and the nitrogen patch (light grey). The plants were pre-treated with a zero background solution only with no phosphorus or nitrogen. Subsequently they were provided with one of three levels of nitrogen. The expectation was that the plants would proliferate more roots in patch with 0.213 mM phosphorus for higher nitrogen availability, or suppress root proliferation in the nitrogen patch for increasing nitrogen availability.

6.4 Discussion

In the split root setup where the plants received the nutrients they were deficient of, nutrient intake of that nutrient increased. In plants that were limited for nitrogen and had ample phosphorus available, phosphorus uptake also increased when nitrogen was supplied. There are clearly two different mechanisms for nitrogen and phosphorus intake, and more importantly, phosphorus intake regulation is much more controlled than nitrogen intake is. If line L is really the optimal feeding rail, over intake of nitrogen is suboptimal behaviour which I can explain as a long-term strategy. In chapter 5 the plants had a very stable nutrient supply throughout their life cycle. If these plants had activated mechanisms to stimulate uptake of the limiting nutrient, it would not have shown much effect because the limiting nutrient was simply not present. In this split root experiment any investment to stimulate nutrient uptake has an effect because the plants were supplied with the limiting nutrient after three weeks. The uptake capacity of a nutrient is increased when it is limiting (Chapin, 1980; Gusewell, 2004), and with the limited ability of the plant to downregulate nutrient intake when conditioned on a nutrient limiting substrate this overshooting of nitrogen intake is not surprising, and more likely to happen than with phosphorus (Bieleski, 1973). Plants do have a large nitrogen storage capacity in the roots as protein-N which is strategy typical for forage grasses (Volenec et al., 1996). Storing nitrogen in this way would not affect phosphorus intake because even though the assimilation of this nitrogen would cost energy, it does not require phosphorus.

With increasing nitrogen availability I expected an increase in phosphorus intake in the free-feeding system. Even though (Magalhaes and Wilcox, 1983) found that ammonium availability stimulated phosphorus intake, this is not what I found. The same explanation as above would apply, that nitrogen is stored as a strategy to overcome random events like bush-fire or grazing (Volenec et al., 1996). Also, since the nutrients were supplied in such high concentrations, another nutrient or carbon could have been a limiting factor that limited the total biomass increase between the 12.84 and 25.67 mM nitrogen treatments. I found that accumulation of N is much larger than that of P. Phosphorus storage is not found commonly in plants and was only achieved through luxury consumption in one of three grass species in the experiments by Oyarzabal and Oesterheld (2009). This is an important finding because it relates to the difference in uptake mechanisms between nitrogen and phosphorus, and confirms that intake regulation of phosphorus is much better controlled than that of nitrogen. This could in turn relate to life history where large nitrogen availability is highly variable and phosphorus availability more constant (Robinson, 1994).

Root proliferation did not show the expected results like Drew (1975) demonstrated. The difference between the local regulation response and the regulation at the system level could be important to explain this discrepancy (Forde and Lea, 2007). Where I expected exclusive root proliferation in the patch containing the limiting nutrient, the root biomass responses were better defined for the total root biomass, where total root proliferation was higher when plants were supplied with the limiting nutrient, irrespective of location. I suggest that a direct parallel with the experiment of Drew (1975) can not be made because, as I explained in chapter 2, the reference to what concentration is a control, the relation with the nutrient state of the plant, the species and the life stage are all factors that influence root proliferation response. Also, the method of nutrient application could have inhibited a root proliferation response, as it did not allow for a depletion zone to develop around the roots. The lack of increase in phosphorus intake with increasing nitrogen availability explains the lack of root proliferation response in figure 35. The increase in nitrogen uptake is still not fully explained as the assumption is that nutrient uptake costs carbon (Fisher et al., 2010) which would be wasteful if there is plenty of this nutrient available. To verify this nitrogen uptake in luxury consumption when a root proliferation response is not observed, a labelled carbon experiment could provide insight in the carbon allocation towards the uptake of nitrogen.

7 Split root experiment - PETIS

7.1 Introduction

From chapter 5 and 6 I concluded that phosphorus intake is well regulated up to amounts that would allow the nutrient intake ratio follow to the optimal feeding rail, and is not taken up in excess. Nitrogen intake exceeded what was expected and high soil solution concentrations caused accumulation in the plant, without proportionally increasing biomass production and stimulating phosphorus intake. Phosphorus availability should be relatively stable among environments, while nitrogen concentrations can vary three-fold on a distance of three centimetres (Robinson, 1994).

Williams et al. (1991) conducted a carbon partitioning experiment in split root systems of barley plants. Carbon partitioning between plant organs can be based on the water relations of sink tissues (Huisinga, 1979; Lang and Thorpe, 1986). The flux into a sink is proportional to the phloem turgor gradient between the source and the sink:

$$J = \frac{\triangle\psi_p}{l}K \tag{22}$$

where:

J = the flux,

$\triangle\psi_p$ = the difference in turgor pressure,

l= the length of the phloem and,

K = a constant dependent on the sieve-element pore dimensions and phloem content viscosity.

So if two equidistant sinks, say two root tips, are importing assimilate provided by the shoots, the greatest flux will go to the root with the lower turgor (Williams et al., 1991). Local supply of nutrients activate the nutrient assimilation processes which requires energy provided by metabolism of assimilates, and the inorganic nitrogen is assimilated into amino-acids and transported into the xylem (Schjoerring et al., 2002). This reduces the turgor pressure, driving the greater partition of the photosynthate flux to travel to this site of depletion.

This leads to the following hypothesis: if carbon allocation partitioning is driven by turgor pressure, the carbon allocation response is local and specific to the site of nutrient assimilation.

This response is direct and on a short time scale which explains the discrepancy with my previous finding in figure 34 and 35. The response on the system level in terms of root proliferation which would affect the root mass fraction is a long-term strategy related to the integrated demand for all nutrients (Mankin and Fynn, 1996). Short-term and long-term are arbitrary terms, in this situation I consider minutes to hours a short-term response, and days to weeks the long-term. In previous chapters I have measured the long-term responses on the system level which are related to the plant's nutrient demand, in this chapter I measure the short-term response to application of the limiting nutrient. To confirm that nitrogen assimilation provides a direct carbon cost at the intake location, which could have been the cause of the lower reproductive success for high nitrogen intake relative to phosphorus, I used ^{11}C as the source for the plant to produce carbohydrates and used the positron emitting tracer imaging system (PETIS) to follow allocation of these carbohydrates on a time-scale of 2 hours.

Naturally occurring carbon is in the isotopes ^{12}C and ^{13}C which occurs in a ratio of 1:99, and ^{14}C which occurs in trace amounts. Low energy β^{-}radiation of ^{14}C is often used to trace carbon allocation, but because of self-absorption by the plant tissue the images have very low resolution (Nakanishi et al., 1999). An improvement for image quality is the use of ^{11}C, which is an artificial radio isotope with a half-life of 20.4 minutes and radiates high energy β^{+} . The positrons annihilate upon encountering an electron which causes two high energy photons to travel in opposite directions (γ radiation). The photons can then be detected by coincidence counting by the detectors which are located on both sides of the plant, from which the exact location of the coincidence event can be calculated (Minchin and Thorpe, 2003). The positron emitting tracer imaging system was developed by the Hamamatsu Photonics Co in collaboration with the TIARA group of the Japan Atomic Energy Agency. This imaging system has a spatial resolution of 1 - 2 mm and can resample every 5 seconds (Keutgen et al., 2005; McKay et al., 1988). This setup allows to measure the relative allocation of the ^{11}C - carbohydrates in the entire body of small plants, which was required for my experiment.

7.2 Methods [3]

Germination I used the same model species as for all the previous experiments, *Poa annua.* The seeds were germinated on filter paper, after two weeks the seedlings were placed in a split-root setup, on a sand/vermiculite substrate and received a low concentration Long Ashton solution.

Split root pots I built the split root pots by glueing together perspex plates separated by plastic bars, and placed a bar in the middle to divide the root zones (for photos see Appendix B.2. These pots were only 1.5 cm thick as to create a rhizotron situation with the roots growing against the perspex plates. I filled the pots with a 50/50 vermiculate and sand substrate up to a total weight of 230 +/- 5 g with the moisture content kept at 60% of water capacity.

Growth conditions I placed the pots at a 70º angle to allow the roots to grow against the perspex. The day-length was set at 16 hours, light was given with daylight temperature artificial light, the temperature was constant at 30º C.

Nutrition All the plants received the same pre-treatment. The first four weeks the plants received 1.604 mM Nitrogen with the equivalent (20%) background solution in 5 ml, twice weekly. The nutrient concentrations, including nitrogen, were adjusted on the basis of plant size and the preferred plant size on the day of the experiment (4 leaf stage), so in the 5th week I increased the concentration to 3.209 mM, adding 5ml to each side of the root system (10 ml in total), two times per week. In week 6 I doubled the nutrient concentrations again to 80%, with 6.418 mM nitrogen and applied it in 5 ml on each side of the roots system.

A week before the labelling experiment I stopped applying nitrogen to the plants. They continued receiving the background solution of 100% strength, 10 ml per plant, every three days. Four days before the labelling experiment the plants were transported to the research station in Takasaki to allow for acclimatisation before the labelling experiment.

On the day of the labelling experiment I selected six plants to make three pairs of similar plant size and visible roots. Because the experiment was at mid-night, the day and night time

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[3] The experiment was facilitated by the Department of Radiation-Applied Biology of the Takasaki Advanced Radiation Research Institute, under the supervision of principal researcher Dr. Shu Fujimaki.

was gradually shifted by changing it by two hours over several days. The labelling experiment started at 01.00 am, so daytime was started at 00.00am.

Labelling experiment Carbon-11 was generated in the cyclotron facility of the Takasaki Advanced Radiation Research Institute. $^{11}CO_2$ was produced by bombarding nitrogen gas with a 10 MeV proton beam. The resulting $^{11}CO_2$ was stored into a stainless steel pipe that was cooled with liquid nitrogen. To ensure consistent supply of 150 MBq, the radioactivity was measured and the timing of supply could then be calculated from the decay rate ^{11}C, which has a decay rate of 20.4 minutes.

The positron emission tomography imaging system (PETIS) consists of two plates detecting gamma radiation (figure 36), from which the exact location of the collision event of the positron with an electron can be calculated. Determination of every emission point allows to construct a static image of the tracer distribution. This system is connected with the computer software that records this information at intervals of 1 minute. The data was translated into Bq, cumulative disintegrations per second.

A a clear acrylic air tube with a valve for the $^{11}CO_2$ inlet and an outlet for the exhaust was placed over the plant leaves and sealed with polyurethane rubber to prevent leaking. Every plant received a dose of 150 MBq over the course of 2 hours. The flow rate was controlled using a supply combination of a compressed CO_2 gas cylinder, an air pump and a mass flow controller. The first 10 minutes the output was passed through a pot of soda lime to collect all the residual $^{11}CO_2$. Throughout the experiment the plants received continuous light from a LED.

I used three replicates, that is 3 runs of a treatment plant and a control plant every two hours. One hour before every carbon application I applied 0.4 mM nitrogen in 5 ml to each side of the control plants root system. The treatment plant received 0.4 mM nitrogen on one side, and 8 mM nitrogen on the other side of the root system. The data reflect the total cumulative disintegrations per second (Bq). Regions of interest could be specified to allow separate calculations for each root partition. Photos of this labelling experiment can be found in Appendix B.2.

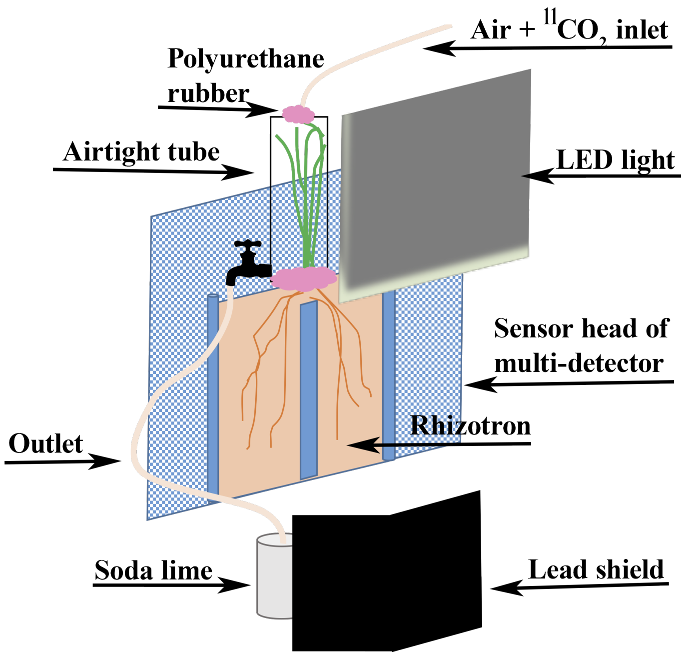


Figure 36: The schematic view of the experimental system, a gas conditioning system for $^{11}CO_2$ feeding to test plants in controlled conditions. The $^{11}CO_2$ is fed into the top of the air tube in which the plant assimilates the radioactive carbon. Photo-assimilates are translocated throughout the plant and into the root system. The sensor registers the location and activity of the positron emissions in the root system. From the bottom of the air tube the contaminated air flows into a jar with soda lime, which is shielded by blocks of lead. The polyurethane rubber prevents the contaminated air from leaking from the air tube.

7.3 Results

Figure 37 shows the image sequence of the first replicate, with the control plant (a) and the treatment plant (b). The treatment plant received 8 mM nitrogen concentration (5ml) on the right side. The scale reflects the relative amount of carbon that is present. The red area is the tube that contains the plant leaves, from 30 minutes the base of the plants starts to become visible, building up carbon assimilates. In time we see the activity into the root systems increase with some highlights emerging from 70 minutes in the treatment plant.

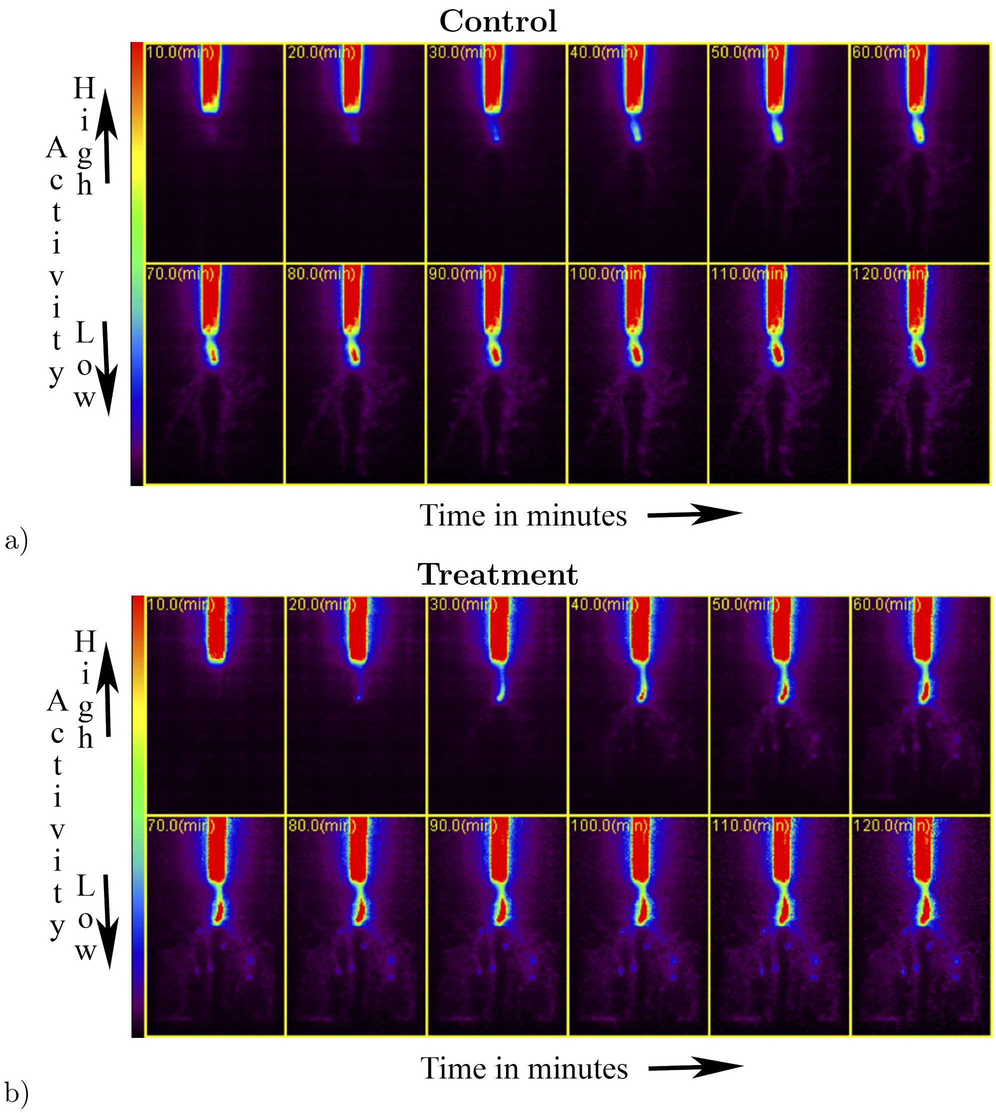


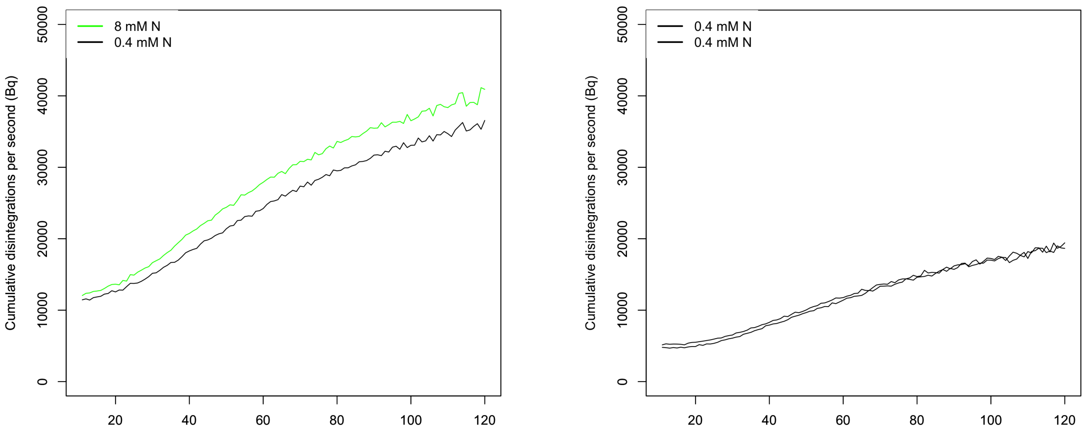
Figure 37: Visual output of the positron emitting tracer imaging system (PETIS). Imaging sequence (per 10 minutes) of the control plant (a) and the treatment plant (b) of the first replicate. High levels of recorded activity are shown in red, whereas low levels are black. The red tube shape shows the tube containing the plants shoots, under which the plant base can be seen through captured carbon activity. Below this base, roots were separated into two compartments by a perspex barrier in the middle, each side receiving either control nutrition, or treatment nutrition. The control plant received 0.4 mM nitrogen on both sides, while the treatment plant received 5 ml 8 mM ammonium long ashton solution on the right side of the root system and 0.4 mM nitrogen on the left side. The roots become visible by the translocation of assimilated labelled carbon into the root system, where the roots become brighter more carbon is allocated to this area. .

Table 23: Activity data in the root partitions

		Treatment	Carbon allocation (Bq)		
		Nitrogen concentration	Replicate 1	Replicate 2	Replicate 3
Treatment plant	Root partition	0.4 mM	36553.6	24382.4	24497.5
	Root partition	8 mM	40909.0	28505.7	28283.1
Control plant	Root partition	0.4 mM	18651.4	23876.7	12506.3
	Root partition	0.4 mM	19425.7	28435.2	16301.1

The results of the three cycles are shown in figures 38, 39 and 40. Each figure represents a pairs of three replicates, where only the treatment side of one of the pair was given the 8 mM nitrogen concentration (in green). The first and third replicate (figure 38 and 40) show what would be expected, that there is a higher activity measured in the treatment side than in the low nitrogen side, and the total carbon uptake seems to be higher than in the control plant. The second pair showed a less convincing response, where the carbon partitioning in the control plant is similar to that of the treatment plant.

To make statistical comparisons, I used the final activity levels of each plant, per root part, and tested for differences between the root compartments. Carbon allocation was directed to both root partitions, with a higher activity measured on the treatment sides than in the low nitrogen side, and no difference between the compartments of control plants (table 24). There were only three data points to compare, which tested to be normally distributed with the Shapiro-Wilk test for normality. Therefore I did a paired t-test for analysing the differences in activity between the root partitions. The total carbon assimilation in the treatment plants was not significantly higher than in the control plants (p=0.064).



a)

b)

Figure 38: Replicate 1 of the Carbon-11 experiment. The y-axes represent the radiation in becquerel as a result of carbon-11 assimilation in the root system. Plot a shows the carbon assimilation of the treatment plant. The two lines show the accumulation in the two root systems. In plot b the same setup is shown with control treatments.

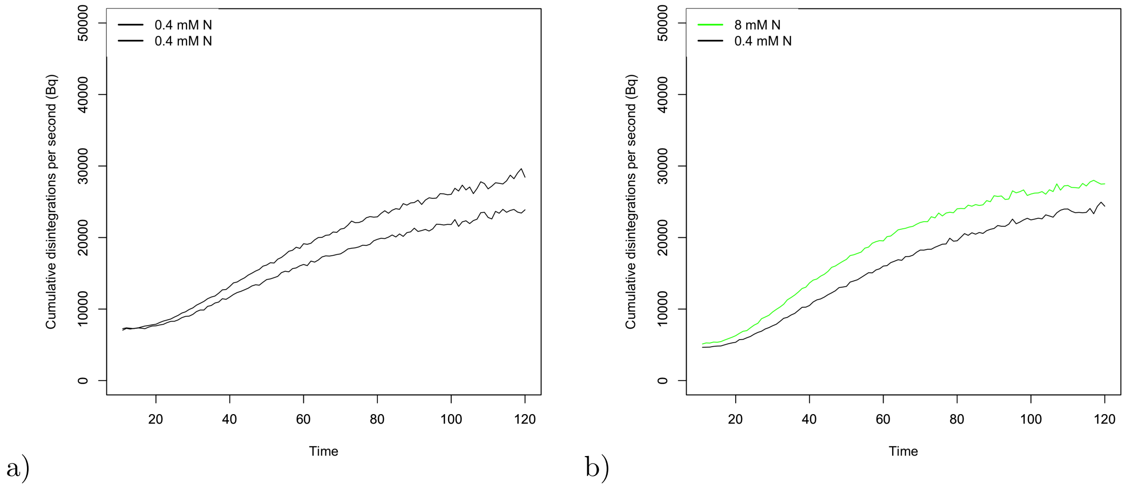


Figure 39: Replicate 2. Even though the carbon assimilation in the treatment plant (b) in the root system receiving 8 mM nitrogen is higher than the side receiving 0.4 mM nitrogen, the control treatment (a) shows the same variation with equal nitrogen availability.

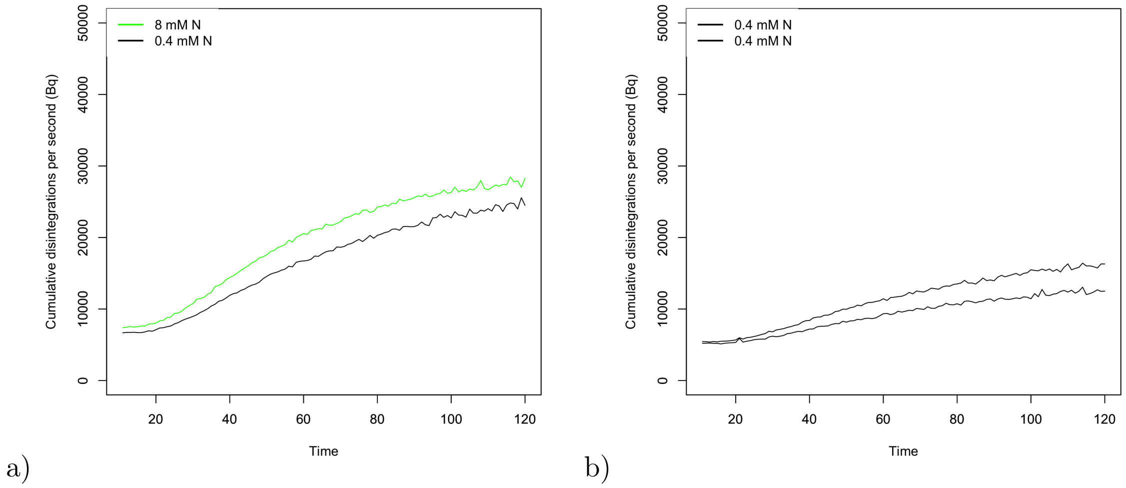


Figure 40: Replicate 3. This plot shows a similar response as replicate 1 in figure 38. The treatment plant (a) accumulates more carbon than the control plant (b). More carbon is allocated to the root system receiving 8 mM nitrogen than to the side that received 0.4 mM.

Table 24: Carbon allocation statistics

	Statistic	Value
Treatment plants	t	-10.547
8 mM, 0.4 mM	df	2
	p	< 0.01
Control plants	t	-2.6335
0.4 mM, 0.4 mM	df	2
	p	0.119

Statistics were calculated with paired t.tests.

Treatment samples one-sided. Control samples 2-sided.

Each three replicates had a normal distribution (Shapiro Wilk test for normality)

7.4 Discussion

I examined the immediate effect of nitrogen application to part of the root system of nitrogen deficient plants on the carbon allocation to the roots. The $^{11}CO_2$ that was pumped into the tube over the leaves was assimilated and transported down the root systems. The time series of cumulative disintegrations per second (Bq) per root partition showed two replicates with clearly higher carbon allocation to the site of high nitrogen availability. A paired t-test showed a significant increase in carbon allocation to this side. The total carbon assimilation in the treatment plants was not higher than in the control plants. Because the plants were nitrogen deprived prior to the labelling experiment, I think that also the 0.4 mM application caused a carbon allocation to the roots system, where the total response is of equal size because it is at its maximum carbon assimilation rate. With equal nitrogen assimilation at the roots there would be no partitioning, whereas the higher turgor pressure at the site of high nitrogen concentration caused the carbon flow to be higher than in the lower nitrogen side.

The difference with the long term biomass investment is interesting and confirms the response on the system level to be driven by the balance of demand for all nutrients and is therefore not partitioned. This labelling experiment has shown that the direct carbon allocation response is directed towards the site of nutrient application, hence the site of highest carbon demand.

With these findings I have shown a direct foraging response in terms of carbon allocation for nutrient assimilation. I can not draw any quantitive conclusions because the carbon responses are not proportional to the difference in nitrogen availability, which is likely limited by the maximum carbon assimilation rate.

Part IV

Conclusions

8 Summary of the Conclusions

In chapter 3 I laid out the hypotheses that I was going to test and the questions that I wanted to answer. In the first experiment I conducted, which was described in chapter 4, I familiarised myself with the model plant *Poa annua*, and although I did not structurally vary temperature and water application, it gave it a good idea of how much watering the plant needed, and it made me decide to increase the temperature and the day-length in the second experiment in an attempt to shorten the life cycle. The more specific findings were the growth responses to the nitrogen and phosphorus availabilities. This biomass data showed that the range of 0 to 6.42 mM nitrogen in the Long Ashton solution, did not cover a maximum biomass production, the linear increase in biomass did not level off and therefore I decided that a better nitrogen regime would be found for higher concentrations. For phosphorus I found a steep increase in biomass production between 0 and 0.27 mM phosphorus concentration, which were the lowest two treatment concentrations. Higher concentrations did not produce any further increase or decrease in biomass. To specify from which point the plant performance in terms of biomass would level off, I chose the range between these two values for the second experiment.

In Chapter 5 I implemented the findings of the first experiment and made some practical changes to improve efficiency. I used a longer day-length and increased the day and night temperatures to reduce the life cycle. With the new ranges for nutrient concentrations I grew the plants again, and with more replicates I was hoping to overcome the high variation in the data that I encountered earlier. The ultimate data of interest were the reproductive success which I calculated as the number of viable seeds per plant, the nitrogen intake and phosphorus intake amounts. These three variables were used to construct the geometric framework for *Poa annua* and to define the optimum intake levels in relation to reproductive success to define the intake target in the fitness landscape.

With this model I could answer the first three questions:

1. Is there a defined target intake for nitrogen and phosphorus for *Poa annua* and if so, where in the nutrient space is it?
   Yes, with the generalised linear model relating reproductive success to nutrient intake, the optimal intake values could be predicted which were located around 130 mg nitrogen and 25 mg phosphorus over the course of 6 weeks.

2. Is there an interaction between the intake nutrients on the fitness cost?
   Yes, the change in fitness with N intake is not equal for all P, and the change with P is not equal for all N.

3. How do the relations between intake and fitness cost, and any potential interactions shape the fitness landscape?
   The area that defines the optimum is a tilted ellipse which means that the cost of nitrogen deficiency can be alleviated by a surplus of phosphorus and vice versa.

- Hypotheses:

1. If plants have evolved to optimise fitness to a defined nutrient intake, there is a cost related to both under and over ingesting that nutrient.
   Both the landscape as described by the model and the observed data that was used to combine the model, showed a decline in reproductive success for high nitrogen intake relative to phosphorus intake.

2. Both Betrand's rule and the marginal value theorem predict a fitness relation to nutrient intake that is not linear, hence the fitness landscape will be an either symmetrical or asymmetrical quadratic tilted shape.
   The relation between fitness and nutrient intake was not linear, the fitness landscape follows a symmetrical quadratic tilted ellipse around the optimum, except for extremely phosphorus deficient situations.

In chapter 5 I was able to establish the optimal feeding rail describing the ratio of phosphorus and nitrogen the plant should take up when absolute optimal intake to reach the intake target was not possible. This line L formed the basis of the third part of this thesis, which was further separated into chapter 6 and 7. In this part the goal was to test whether a foraging response could be observed as it was in (Drew, 1975), in the proportions of the nitrogen and phosphorus demand. The demand was manipulated to be for either nitrogen or phosphorus exclusively, and the roots shoot forage accordingly. I did a split root experiment which would mimic the presence of different nutrient patches and I measured the nutrient intake and the root investment in each of those patches. Also, with high nutrient concentrations but in different ratios I tried to mimic a free-feeding system to test whether root investment would be regulated such that the intake ratio would be stable and matching the optimal feeding rail.

The intake data showed interesting differences between nitrogen and phosphorus, where phosphorus intake was well regulated and nitrogen intake was higher than expected and related to nitrogen availability, even though we saw earlier that this incurs a fitness cost. To distinguish between foraging response on the long term, on the system level and based on nutrient demand and the response on the short term, local level and based on the physical interactions, I did an experiment with radioactive ^{11}C that showed the carbon allocation response in the first two hours after nitrogen application of a nitrogen deprived plant. These two experiments provided the information to answer the final four questions:

1. Is exclusive feeding observed in any nutrient state where $\triangle(N,P)\neq 0$ in the model species *Poa annua?*

   When the plant is phosphorus deficient, the plant takes up phosphorus exclusively when provided with it. When the plant is nitrogen deprived, it did not take up nitrogen exclusively and increased both its nitrogen and its phosphorus status.

2. Is nutrient intake regulated by exclusive root proliferation into the resupplied patch of the deficient nutrient?

   Nutrient intake was not regulated by exclusive root proliferation, total root proliferation was higher in the plant provided with the deficient nutrient but there was no partitioning between patches.

3. Can the intake response to nutrient supply of the deficient nutrient directly be observed as a carbon allocation response?

   Yes, the direct response of nitrogen deficient plants to nitrogen application to part of the root system, is to allocate a higher amount of assimilated carbon to the site with high nitrogen supply.

- Hypotheses

1. If nutrient intake selection depends only on the proportion of each nutrient in the available solutions and its effect on state change, and the state change $\neq 0$, the optimal strategy is to maximise or minimise λ, hence, take up one nutrient exclusively.

   This hypothesis may be true depending on the nutrient under study, and the life history of the plant and environmental variability related to the availability of that nutrient. Regulation is also restricted to the abilities of the plant.

2. Root proliferation, root hair growth, transporter protein activation and nutrient assimilation require carbon, hence both root proliferation and carbon transport to the root system is exclusively directed to the patch containing the deficient nutrient.

   Exclusive root proliferation was not observed, whereas higher carbon allocation to the patch containing the deficient nutrient was found as a direct and site specific response.

Epilogue

The geometric framework as presented in this thesis could be used in future studies on plant root foraging behaviour. It is essential to understand the uptake mechanisms and interactions of the nutrients under study, to define the expectations with respect to the fitness landscape. The use of strictly controlled growth facilities contribute to the reduction of variation in the data and the strength of the defined intake target. When defining optimal intake targets and ratios, reproducibility is key to ensuring that the behaviour of different species can be compared. Hence, I suggest the geometric framework allows the range of nutrient intake to be specified with the optimum as the calibration point. I studied the behaviour of individual plant exclusively and in controlled growth conditions. Plant however have evolved and developed optimal strategies in the field. Therefore the geometric framework could be extended by integrating the interactions that plants would experience in the field such as competition and symbiosis. Extensive research has been done on root foraging behaviour in competition with other plants, and it would be an interesting next step to bring this knowledge together with the theoretical framework presented here. Also, the symbiosis with mycorrhiza for example would be an essential aspect to integrate with the geometric framework since this symbiosis is invaluable for plants in the field.

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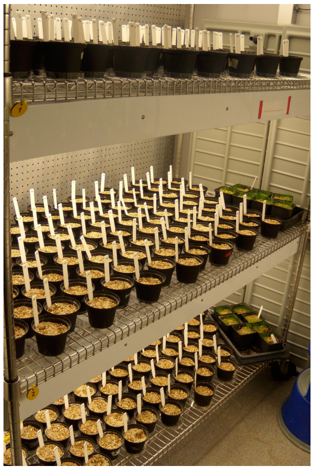
Appendices

Appendix A: Defining the optimal nutrition of *Poa annua*

A.1 Photo's Chapter 4

Seedlings, several days after germination.

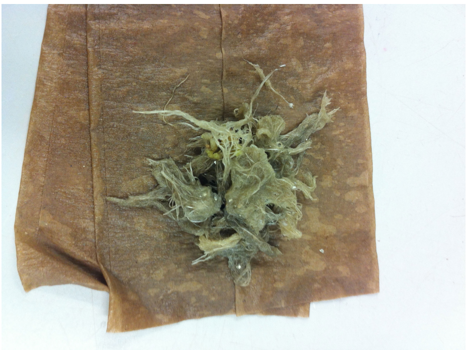
Flowering plant, typical for low to intermediate nutrient concentrations.



Controlled growth chamber.

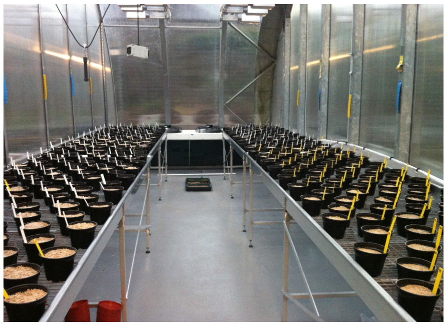
Preparation of Long Aston solutions.

Seed collection.



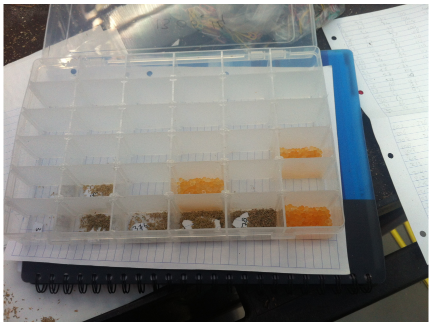
Washed root system of one plant.

A.2 Photo's Chapter 5



Randomised placement

in the greenhouse.

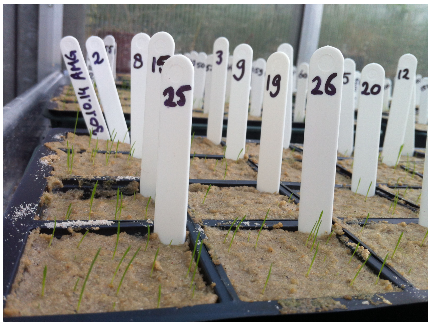


Seed storing system with

silicate gel beads.

Seeds from a typical nitrogen and

phosphorus sufficient plant.



Seed germination to calculate the

number of viable seeds, 25 seeds

per square, placed in a 5x5 grid.

Appendix B: Testing the two-food model

B.1 Photo's Chapter 6:

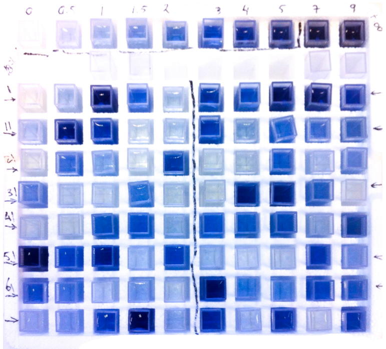
In this split root experiment I used pots that I made by taping sandwich bags around a cylinder up to the exact desired volume. These pots were kept together with a tie wrap and the bottoms were pierced to allow for drainage. Plant material was acid digested by heating it in sulphuric acid up to 365 ºC. The colorimetric determination is a method based on creating a standard colour gradient related to a phosphorus (blue) or nitrogen (green) concentration, and matching the colour of the sample material to the standard.

Split root setup. Flexible plastic pots with pinched holes at the bottom.

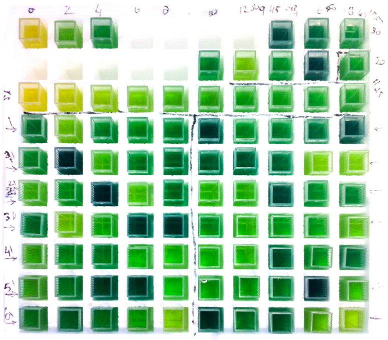
Root system in a nitrogen supplied patch.

Clean root system of a plant grown in a split root setup.

Acid digestion of the plant material.



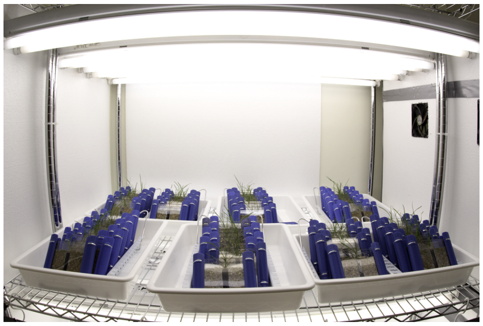
Colorimetric determination of phosphorus (example). The standard curve is plotted from the top row.



Colorimetric determination of nitrogen (example). The standard curve is plotted from the third row.

B.2 Chapter 7 C-11 labelling experiment photo's

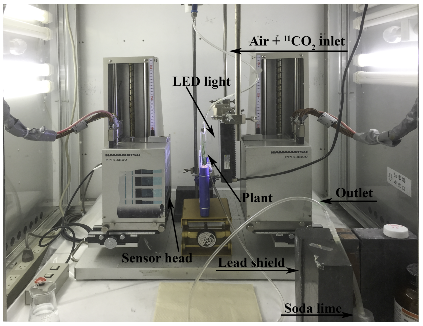
The Positron Emission Tomography Imaging System (PETIS) setup is shown here. The plants were grown at an angle in flat rhizotrons pot to see the roots grow against the perspex. An airtight tube was placed over the leaves in which the carbon-11 was injected.



Plant preparation
with pre-treatment.

Installation for the
PETIS experiment.

The airtight tube is placed over the leaves and
sealed with polyurethane gum.



Complete experimental
setup for the PETIS imaging.

The end